STUDIES

FROM

THE ROCKEFELLER INSTITUTE

FOR MEDICAL RESEARCH

REPRINTS

VOLUME 139

NEW YORK
THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH
1949

Made in the United States of America

Results of the investigations conducted at the Rockefeller Institute, or elsewhere under its grants, are first reported in a variety of journals and publications. The reports are then assembled in volumes designated Studies from The Rockefeller Institute for Medical Research, of which this is Volume 139. The Studies appear serially but at irregular intervals. The text of the original publications is in all respects followed in the Studies. The name, date, volume, number, and pages of the journal in which each article originally appeared are printed above the title. To insure uniformity and simplicity of reference, plates and illustrations repeat the numbers used in the place of first publication.

CONTENTS

THE DEPARTMENT OF THE LABORATORIES

Chemistry

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, March, 1949, Vol. 178, No. 1, pp. 53–77]

CHROMATOGRAPHY OF AMINO ACIDS ON STARCH COLUMNS. SOLVENT MIXTURES FOR THE FRACTIONATION OF PROTEIN HYDROLYSATES

BY STANFORD MOORE AND WILLIAM H. STEIN

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, October 8, 1948)

In previous communications (1–3) procedures have been described for the quantitative separation of amino acids by chromatography on starch. The present paper is concerned with the extension of these techniques to include most of the amino acids commonly found in protein hydrolysates. In the earlier experiments *n*-butyl alcohol-benzyl alcohol solvents containing about 15 per cent water were employed to separate phenylalanine, leucine, isoleucine, methionine, tyrosine, and valine. In all alcohol-water solvents these are among the fastest moving amino acids on starch columns. Preliminary experiments had indicated (1) that the amino acids with slower rates of travel could be eluted successfully from the column by the appropriate choice of acidic solvents of higher water content. Many types of solvents have subsequently been investigated in order to arrive at a convenient system for the fractionation of protein hydrolysates.

The effluent concentration curves shown in Figs. 1 and 2 give the results obtained with two of the solvent mixtures which have proved most useful. The synthetic mixture of amino acids chromatographed corresponded in composition to a hydrolysate of bovine serum albumin. The effluent from the column was collected in a series of 0.5 cc. fractions on an automatic fraction-collecting machine (2). The amino acid concentration in each fraction was determined by the photometric ninhydrin method previously described (3). For the curve in Fig. 1, the column is started with a solvent composed of *n*-butyl alcohol, *n*-propyl alcohol, and 0.1 N HCl in the proportions of 1:2:1. After the emergence of aspartic acid, the rates of travel of the amino acids remaining on the column are increased by a shift of solvent to 2:1 *n*-propyl alcohol-0.5 N HCl. In this experiment, the first six amino acids are incompletely separated, and a chromatogram run with 1:1:0.288 *n*-butyl alcohol-benzyl alcohol-water (2) is still required for resolution of these components.

The curve in Fig. 1 gives quantitative values for proline, threonine, aspartic acid, serine, glycine, ammonia, arginine, lysine, histidine, and cystine. Glutamic acid and alanine appear as a single peak. These two amino acids can be separated by the chromatogram illustrated in Fig. 2. The solvent in this case is composed of *tert*-butyl alcohol, *sec*-butyl alcohol,

and 0.1 N HCl in the proportions of 2:1:1. Thus, by the use of three columns it is possible to separate from one another the eighteen constituents most commonly encountered in acid hydrolysates of proteins. The

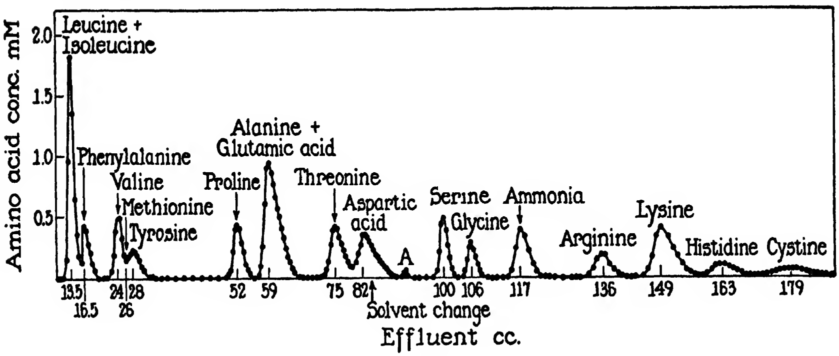


FIG. 1. Separation of amino acids from a synthetic mixture containing seventeen amino acids and ammonium chloride. Solvents, 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1 N HCl, followed by 2:1 *n*-propyl alcohol-0.5 N HCl. Column, 13.4 gm. of starch (anhydrous); diameter, about 0.9 cm.; height, about 30 cm. Sample, about 3 mg. of amino acids. *A* is a small artifact peak (see the text).

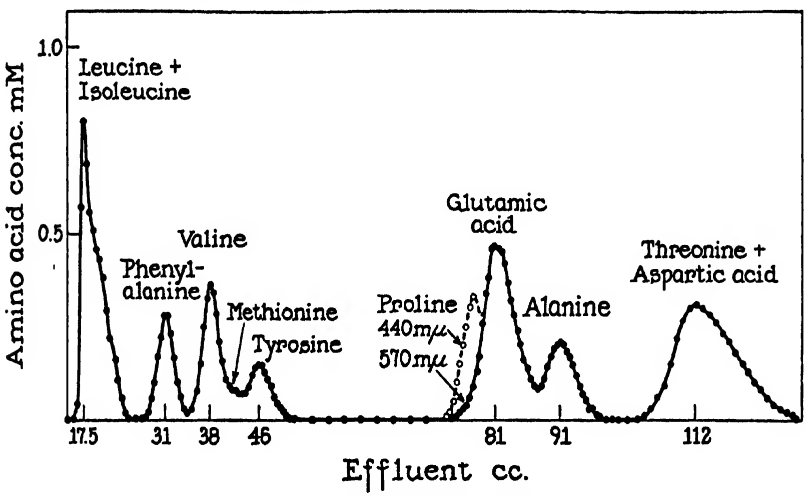


FIG. 2. Separation of glutamic acid, alanine, and other amino acids from a synthetic mixture containing eighteen components. Solvent, 2:1:1 *tert*-butyl alcohol-*sec*-butyl alcohol-0.1 N HCl.

following experimental section describes the procedure employed to obtain results of the type shown in Figs. 1 and 2. The discussion deals with some of the considerations introduced by the presence of additional components

in the mixture being fractionated and outlines the results obtained by the use of other solvent combinations. The results of analyses of hydrolysates of β-lactoglobulin and bovine serum albumin are given in the following paper (4).

EXPERIMENTAL

Preparation of Column—The potato starch column is poured as previously described (2).[1] Unless otherwise specified, starch columns 0.9 cm. in diameter and about 30 cm. in height have been used. The procedures can be scaled up proportionately for columns up to 8 cm. in diameter. After the starch has settled to constant height, the excess butyl alcohol is removed and a 1:1 mixture by volume of *n*-propyl alcohol-0.5 N HCl is placed on the column. The solvent is run through the column under a pressure of 8 cm. of mercury overnight, and the pressure is then raised to 15 cm. The solvent flow is continued until 0.5 cc. samples (neutralized) of the effluent and the influent solvent both yield the same color value when analyzed by the ninhydrin method. Starch contains small quantities of ninhydrin-positive material which are extracted by acidic alcohol solvents. The use of a propyl alcohol-HCl mixture with a 50 per cent water content serves to clean out the column fairly rapidly. When the number of cc. of the effluent required to yield a ninhydrin-negative column have been determined for a given batch of starch, the prescribed number of cc. can be used in the preparation of subsequent columns. For the samples of starch tested thus far, 55 cc. of the 1:1 solvent have proved adequate for columns 0.9 × 30 cm.[2] When the column is ninhydrin-negative, the solvent mixture is changed to that to be used in the chromatographic analysis.[3] After the new solvent has been run through overnight at 15 cm. pressure (20 to 25 cc. of effluent), the column is equilibrated and ready for use. Columns may be left in contact with solvents of low acidity,

[1] For work with acidic solvents, the delivery tip of the chromatograph tube can be pulled down so that a drop of effluent collects therein. In this manner ammonia from the air is prevented from reaching the inner walls of the tip. Beveled tips are still required on tubes which will be used with water as the solvent. If a beveled tip is used with acidic solvents, the inside section, up to the sintered plate, must be rinsed with a stream of the solvent before the column is placed on the fraction collector. A pipette, the end of which has been bent to form a U, is used for the rinsing.

[2] It is possible to wash large amounts of starch at one time with the propyl alcohol-HCl solvent, thus avoiding the preliminary washing each time a column is poured. This procedure is not recommended, however, since samples of starch washed and dried in the laboratory have been found not to give as uniform columns as the untreated commercial material (2).

[3] The solvents employed in these investigations have been prepared from *n*-butyl alcohol (reagent grade, Merck) and *n*-propyl alcohol, *sec*-butyl alcohol, and *tert*-butyl alcohol (C.P. grade, Columbia Organic Chemicals Company, Inc., Columbia, South Carolina). Redistillation prior to use has not been found necessary.

such as those prepared from 0.1 N HCl referred to in Figs. 1 and 2, for about 2 weeks before use without deterioration. Prior to the addition of the sample, the surface of the column is packed as previously described (2). When acidic solvents are used, there is no need for the 8-hydroxyquinoline treatment, which has been shown to be essential when neutral solvents are employed (2). It is desirable to run about 0.5 cc. of solvent into the freshly packed surface before the addition of the sample.

Addition of Sample to Column—The synthetic mixture of amino acids employed in the experiments shown in Figs. 1 and 2 was made up to simulate an acid hydrolysate of bovine serum albumin. To a total of about 1 gm. of amino acids in a 10 cc. volumetric flask, 1.5 cc. of 6 N HCl were added and the solution diluted to volume with water. A 0.5 cc. aliquot of this solution was diluted to 10 cc. with the solvent to be used in the chromatogram. A 0.5 cc. aliquot of the final solution, corresponding to 2 to 3 mg. of the amino acid mixture, was placed on the column and washed in as described earlier (2). In the developmental work on the placement of the peaks, simpler mixtures containing only a few components were similarly prepared. The pipettes should be calibrated for delivery both with water and with the organic solvent mixture.

Collection of Effluent Fractions—The delivery tip of the chromatograph tube is cleaned with a moist cloth and the column is placed on the automatic fraction collector (2). The pressure is maintained at 15 cm. and 0.5 cc. fractions are collected. The flow rate on a properly packed column should be 1.25 to 1.50 cc. per hour.

The use of propyl and *tert*-butyl alcohols on the fraction collector introduces problems which were not encountered with the butyl or benzyl alcohol solvent mixtures investigated earlier. When 0.5 cc. samples of the more volatile alcohol mixtures are allowed to stand on the machine overnight, there is considerable evaporation from the tubes. The loss in volume is not important, since the entire fractions are used in the ninhydrin analysis. But it has been noted that propyl alcohol-water mixtures, for example, have a marked tendency to creep up the glass walls of the photometer tube during the process of evaporation. Within 18 hours the solvent may creep almost to the top of the tube. The process can be observed by dissolving a few crystals of methyl red in 0.5 cc. of 2:1 *n*-propyl alcohol-0.5 N HCl. The quantity of amino acid which is carried to the upper portions of the tube as the solvent evaporates may comprise 4 to 8 per cent of the total amount present. This material is not in contact with the ninhydrin reaction mixture during the analytical determination. Hence, the recoveries of amino acid, under these conditions, run low.

It has been found that the creeping of volatile alcohols can be completely eliminated by rendering the glass surface hydrophobic by means of a

silicone film. Glassware coated with a silicone film is repellent to water and to the water-miscible alcohols such as propyl alcohol and *tert*-butyl alcohol. For the present experiments all the sets of photometer tubes (3) for use with the fraction collector have been coated with Dri-film No. 9987 (General Electric Company, Schenectady, New York), which is a mixture of organochlorosilanes. For polymerization on glass, the Dri-film is applied as a 5 per cent (by volume) solution in chloroform (reagent grade). The coating of the tubes should be carried out in a hood and gloves should be worn. The glassware is first cleaned in chromic-sulfuric acid cleaning solution, thoroughly rinsed, dried at 110°, and allowed to stand at room temperature for 1 hour. When sets of 200 tubes are being coated, 200 cc. of the Dri-film solution are prepared. A sheet of filter paper or a towel is placed on the bottom of each test-tube rack (3). The first ten tubes are filled about half full with the filming solution. Each tube is emptied rapidly over a flask or beaker, causing the solution to flow over the upper walls of the tube, and set to drain inverted in the rack. The filming solution is used over again for the treatment of 200 tubes. The racks are left at room temperature overnight. The tubes are then returned to the upright position and each rack is baked for 2 to 3 hours in an air oven at 150–180°. This procedure has given more durable silicone films than those obtained by applying the Dri-film in vapor form or by the use of less concentrated solutions of the coating agent. Control of the relative humidity at which the filming is conducted has not proved necessary.

The film has no effect on the optical properties of the tubes as measured in the Coleman junior spectrophotometer. The silicone-coated tubes have maintained their water repellency during constant use for periods of about 6 months, at the end of which time recleaning and refilming have been necessary. The film is remarkably resistant to boiling water, alcohols, or acids, but is readily destroyed by alkali or cleaning solution. The coating is also rendered ineffective by ordinary soap, but Duponol C has been found to have no injurious effect. The washing procedure for the coated photometer tubes, therefore, is different from that previously described (3). After each set of ninhydrin analyses, the tubes are rinsed with water in racks of 50 and scrubbed with a brush (*e.g.*, E. Machlett and Son, New York, catalogue No. A-7-870) which has been dipped in a 0.2 per cent solution of Duponol C. The brushing is necessary to remove the ring of material that is sometimes deposited on the walls of the tubes. If this deposit resists removal by brushing, it is an indication that the tubes need refilming. An aluminum rod notched to fit the rim of the tube is useful for holding the individual tubes in position in the rack while they are being brushed. The brush employed should be reserved for this purpose and kept out of contact with ordinary soap. No evidence of any scratching of

the photometer tubes by this cleaning procedure has been observed, but care should be taken to insure that no metal parts of the brush make contact with the walls of the tubes. The tubes are rinsed several times with distilled water and dried in an oven at 110°.

To prevent creeping of the solvent on the tip of the chromatograph tube and the glass funnel of the fraction collector, these items are also given a silicone coating. The tip of the chromatograph tube is cleaned with a hot mixture of HNO_3 and H_2SO_4 and coated by dipping the lower portion of the tube in the Dri-film solution, contamination of the sintered glass plate being avoided. The funnel of the fraction collector is coated both inside and outside.

In order to be certain of the proper setting for the impulse counter when a water-repellent tip is used, it is necessary to redetermine the drop size (2) more frequently than is required with an untreated funnel. For the solvents referred to in Fig. 1, the drop sizes have been so nearly the same that a single impulse counter setting has been used throughout the experiment.

The use of acidic solvents requires precautions against the uptake of ammonia from the air by the effluent fractions during the period they are standing on the automatic fraction collector. The ninhydrin method employed to analyze the effluent can readily detect 0.1 γ of ammonia per cc. If no preventive steps are taken, tubes containing 0.5 cc. of 2:1 propanol-0.5 N HCl, left overnight open to the laboratory air or on the fraction collector, may pick up enough ammonia to give a positive reading of 0.10 optical density unit in the ninhydrin analysis. This uptake may be virtually eliminated by lining the inside surface of the cover of the fraction collector with filter paper impregnated with citric acid. Large sheets of filter paper are cut to fit the cover and taped in position. A 2 per cent solution of citric acid in ethanol is brushed onto the surface. With the fraction collectors in use in this laboratory, the ammonia problem has been increased by the liberation of ammonia from the bakelite parts of the machines. It was not appreciated for some time that hexamethylenetetramine is used in the manufacture of many samples of bakelite and that the material, as a result, may contain appreciable quantities of ammonia. Samples of bakelite can readily be tested for ammonia liberation as described earlier (2). If the test is positive, the citric acid solution must be applied to all the bakelite parts of the fraction collector, including the phototube housing. Commercial models of the fraction collector are currently being built with special ammonia-free bakelite,[4] which eliminates this source of contamination.

In work with acidic solvents, the cotton packing around the stem of the chromatograph tube is also treated with citric acid. When the tubes are removed from the machine, they are stoppered with corks which have

[4] The Technicon Company, 215 East 149th Street, New York 51.

previously been shaken with the alcoholic citric acid solution and air-dried. Corks thus treated have been satisfactory for a year or more. Rubber stoppers have proved unsuitable.

Contamination with ammonia can also occur during the handling of the solvents. The lips of all storage vessels should be wiped before use. Care must be taken to avoid any liquid contact between the solvent and the rubber stoppers on the top of the column and the top of the separatory funnel. The glass should always be wiped dry before the insertion of the stoppers. It is important that the need for reimpregnation of the cover on the machine be checked periodically by placing test samples of the 2:1 *n*-propyl alcohol-0.5 N HCl solvent on the machine overnight. The ninhydrin readings should be no higher than those of control tubes which have remained stoppered prior to analysis.

In performing a chromatogram of the type referred to in Fig. 1, a solvent change is made about half-way through the experiment. The effluent fractions should, if possible, be analyzed each day to provide a check on the progress of the experiment and to furnish a basis for estimating the exact point at which the solvent change should be made. In an experiment such as that shown in Fig. 1, it is desirable to shift the column to the second solvent mixture during the emergence of aspartic acid. This point can be predicted fairly accurately by multiplying the position of the readily identified proline peak by 1.6. The change point is usually reached at about 83 cc. of the effluent and can be predicted from the position of one of the earlier peaks, if necessary. If the change of solvent is scheduled to occur at an inconvenient hour, the column can be slowed down by running it under lower pressure without affecting the results. At the time of the change of solvent, the separatory funnel is removed and the liquid above the starch in the chromatograph tube is withdrawn before the addition of the new solvent.

For the experiment illustrated in Fig. 1, the solvent shift occurs on about the 3rd day, and the completion of the experiment, through the emergence of cystine, requires about 7 days of continuous operation on the fraction collector.

When a column is shifted from one solvent to another, a specific series of changes occurs in the composition of the effluent. In the example shown in Fig. 1, the initial solvent contains 25 per cent water and is 0.025 N with respect to HCl. The second solvent contains 33 per cent water and is about 0.17 N with respect to HCl. The effluent attains the higher water content of the second solvent when a volume of solvent equivalent to that retained by the column has passed through the starch. The increased water content, which appears at about 6 cc. after the solvent change, serves to increase the rates of travel of the amino acids. If the solvent shift has been made too early, the latter part of the aspartic acid curve will be

distorted. Since asymmetrical peaks frequently indicate the presence of more than one component, it is preferable, in order to avoid ambiguity, to arrange for the emergence of the higher water concentration after the aspartic acid curve is down to the base-line.

The increase in HCl concentration, however, to 0.17 N, occurs sharply at about 14 cc. after the solvent has been changed. The HCl thus has a "retention volume," in the terminology of Tiselius (5), of about 6 to 8 cc. The rise in the HCl content of the effluent in Fig. 1 occurs just at the beginning of the serine peak. Although a change in acid concentration is not capable of distorting the serine peak significantly, it is desirable from the analytical standpoint to have the change occur before the amino acid emerges.

The peak *A* in Fig. 1 is an artifact which occurs at the point of increase of the water content of the effluent. This small peak represents a transient rise of only 0.02 to 0.04 optical density unit in the blank and indicates that the starch column has been thoroughly freed of ninhydrin-positive material in the preliminary washing procedure. It is indicative also of the adequacy of the silicone film on the tip of the chromatograph tube and the funnel. In earlier experiments, before these parts of the glassware were coated, a relatively large artifact peak was usually obtained at position *A*. A control experiment with a strong solution of methyl red in the acidic solvent demonstrated that, during a 1 to 3 day run on unfilmed glassware, a small amount of solid material was deposited around the outside of the tip of the funnel as a result of creepnig and evaporation of the solvent. Similarly, a deposit of some of the solute could be seen around the periphery of the flowing stream of the effluent within the funnel. When the solvent was subsequently shifted to one of higher water content, and consequently different surface properties, some of this residue was redissolved and emerged as the artifact peak. A similar experiment with glassware rendered hydrophobic by a silicone film showed no residual deposit of methyl red on any part of the tip or funnel.

The shift from one solvent to another, after a sample has been added to a chromatogram, has proved practical only with solvents that are miscible with water in all proportions. When an attempt has been made to shift a butyl-benzyl alcohol column to a propyl alcohol-water solvent, droplets of water have formed at the interface, thus destroying the efficiency of fractionation (2).

Analysis of Effluent Fractions—The concentration of amino acid in the effluent fractions is determined by the photometric ninhydrin method (3). For the 0.5 cc. fractions, 2 cc. of the ninhydrin reagent are used. The solvents possessing a total acidity of 0.025 N or less do not require neutralization. Samples of the 2:1 *n*-propyl alcohol-0.5 N HCl mixture, however, must be neutralized just before the addition of the reagents. For

routine work, a burette tip of appropriate size can be prepared to deliver 0.10 cc. of alkali per 2 drops. A rack of 50 tubes can conveniently be moved along underneath a burette dripping at a constant rate. The rack should be shaken by hand after the addition of the alkali. The concentration of NaOH (about 0.8 N) is adjusted so that, in the titration of test samples, 2 drops leave the fractions slightly acidic. The amount of alkali added should be such that an additional 0.1 cc. of 0.1 N NaOH is required to render the samples alkaline to phenolphthalein. One purpose of keeping the samples slightly acid is to avoid loss of ammonia from the NH_4Cl peak.

After a solvent shift, as in Fig. 1, it is necessary to locate the effluent fraction at which the increase in acid concentration occurs. A small drop of 0.02 per cent phenolphthalein in ethanol is added to the twenty-eighth fraction after the time of change of the solvent on the top of the column. Depending upon whether this tube is or is not rendered alkaline by 1 drop of the approximately 0.8 N NaOH, the tubes ahead or after it are treated similarly until the point is determined at which all subsequent fractions require 2 drops of the alkali. The subsequent additions are made without use of the indicator. By this procedure a few of the fractions around the change point may be overneutralized. No significant errors have been observed when the increase in acid concentration occurs during the first two or three fractions containing serine, but as already mentioned, it is preferable to have the change occur earlier.

For an experiment such as that illustrated in Fig. 1, every effluent fraction is analyzed until after the emergence of glycine. From that point on, analysis of every other fraction is sufficient. If the first chromatogram on an unknown sample shows areas in which there are no peaks, such as the long valley between tyrosine and proline in Fig. 1, the number of analyses required in a duplicate experiment can be reduced by omitting some of the fractions. If the chromatogram is being run for the determination of only one or two amino acids, the rest of the curve can be neglected. The solvent mixture referred to in Fig. 2 is usually employed only to separate glutamic acid and alanine, and generally the first 45 cc. of effluent are collected as a fore fraction before the column is placed on the fraction collector. If accuracy to the last few per cent is not important, the amount of ninhydrin required can be halved by the use of only 1 cc. of ninhydrin solution per 0.5 cc. sample.

The choice of blanks against which the amino acid peaks are read is crucial for maximum accuracy in the integration of the curves. In many instances the average blank tube for the base-line of the effluent curve can be readily determined in the manner previously outlined (2). In the first part of Fig. 1, there are blank tubes ahead of leucine and in the valleys before proline and threonine. The proline peak, reddish yellow in color,

is read at 440 mμ. There is always the possibility, however, that a given group of tubes taken for ninhydrin analysis may not contain an adequate number of fractions from the blank sections of the curve. Therefore, three or more empty photometer tubes are added routinely to each rack of samples submitted to the ninhydrin analysis. The prescribed amount of ninhydrin solution is added to all the tubes. The reagent blank, obtained on the tubes which received only the ninhydrin solution, may vary slightly from day to day or with the batch of reagent solution. The reagent blank frequently amounts to 0.14 to 0.20 optical density unit when read against a reference tube of 1:1 *n*-propyl alcohol-water (3). The column blanks with the 1:2:1 solvent of Fig. 1 and the 2:1:1 solvent of Fig. 2 are usually not identical with the reagent blank, differing by perhaps 0.01 optical density unit. If there is no definite group of column blanks in the set being analyzed, the tubes can be read against the reagent blank. The readings can subsequently be corrected to a column blank by reference to the differential between the column blank and the reagent blank in the preceding or the following day's analyses.

The change of solvent to 2:1 *n*-propyl alcohol-0.5 N HCl introduces changes in the column blank. Following the emergence of the artifact peak (*A* in Fig. 1), the solvent of increased water content which is then emerging may give a reading that is 0.01 to 0.03 optical density unit higher than the reagent blank. An additional rise of 0.01 to 0.02 unit takes place at the point where the increased acid concentration appears in the effluent. The valley between serine and glycine does not always fall to the base-line, and the column blank for both of these peaks is, therefore, taken in the valley after glycine. The fractions before or after the ammonia peak provide the blank in this range. In order to obtain accurate values for ammonia, a standard solution of NH_4Cl and its appropriate blank should be run along with the column samples (3). The base-line for the arginine peak is taken from the valley between ammonia and arginine. The fractions between arginine and lysine usually return to this same value, but not invariably.

It has been found that quantitative recoveries of lysine, histidine, and cystine are obtained only when these amino acid peaks are read against the column blank taken after the emergence of cystine. The valleys in this range do not always fall to the base-line. Since cystine is the last amino acid to emerge, the proper blank is usually not available when the lysine and histidine peaks are analyzed. In this range, therefore, the tubes are read against the fraction giving the lowest reading or against the reagent blank as zero. If some of the tubes have been read against a fraction which gives a reading 0.05 optical density unit above the reagent blank, and the final column blank after cystine has dropped to 0.02 unit above the

reference tubes, the correction is made by adding 0.03 unit to the appropriate fractions before integration of the peaks.

The variations in the blank and the need for the use of these corrections, however, mean that the accuracy of integration of the peaks after the solvent shift in Fig. 1 is, as a rule, not as satisfactory as that obtained in chromatograms developed with a single solvent mixture.

TABLE I

Ninhydrin Color Yields from Amino Acids in Organic Solvent Solutions

Determined on 0.5 cc. samples of 0.35 mM solutions of the amino acids. Heating time 20 minutes. The samples in 2:1 *n*-propyl alcohol-0.5 N HCl were neutralized with 0.1 cc. of about 0.8 N NaOH prior to analysis.

Compound	Color yield on molar basis relative to leucine in water, read at 570 mμ		
	1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1 N HCl (d^{25} =0.862)	2:1:1 *tert*-butyl alcohol-*sec*-butyl alcohol-0.1 N HCl (d^{25} = 0.858)	2:1 *n*-propyl alcohol-0.5 N HCl (d^{25} = 0.882)
Leucine	0.99	1.00	
Isoleucine	1.00	1.02	
Phenylalanine	0.85	0.85	
Valine	1.01	1.02	
Methionine	1.00		
Tyrosine	0.86	0.86	
Proline	0.05 (0.27 at 440 mμ)	0.05	
Glutamic acid	1.02	1.02	
Alanine	1.02	1.00	
Threonine	0.94		
Aspartic acid	0.89		
Serine			0.94
Glycine			0.98
Ammonia			0.98 *ca.**
Arginine			0.97
Lysine			1.14
Histidine			1.01
Half cystine			0.54

* For accurate ammonia determinations the factor should be checked with a known NH_4Cl solution run at the same time as the unknown (*cf.* (3)).

Calculations—The procedure for integration of the curves has been outlined earlier (3). When only every other effluent fraction is analyzed (*i.e.*, ammonia through cystine, Fig. 1), satisfactory recoveries are obtained by doubling the usual summation (*cf.* (3), Table V[5]). For the relatively volatile solvent mixtures referred to in Figs. 1 and 2, the entire 0.5 cc.

[5] Table V (3) contains an error. In the third line of the integration below Table V, read "Sum of Fractions 37–40 and 45–47" for "Sum of Fractions 37–42 and 46–47."

sample evaporates during the 20 minute heating in the water bath. For unneutralized samples, the calculated correction factors for 5, 10, and 15 cc. of diluent (*cf.* (3), Table III) become 0.230, 0.216, and 0.212. For samples which have been neutralized with 0.10 cc. of NaOH, the factors are 0.232, 0.218, and 0.212. In the integration of the curves, the summations of the uncorrected amino acid concentrations are routinely multiplied by one-half the above factors (*cf.* (3), Table V[6]). The whole factors are used for the conversion of the analytical results to leucine equivalents in plotting the curves for publication and in the determination of ninhydrin color yields on standard solutions. If only 1 cc. of the ninhydrin solution is used per 0.5 cc. sample, the evaporation loss is about 0.62 cc. The factors are 0.193, 0.196, and 0.199 for unneutralized fractions and 0.196, 0.198, and 0.199 for samples neutralized with 0.1 cc. of NaOH.

The ninhydrin color yield for each of the amino acids has been determined in the solvent in which it emerges from the column. The yields given in Table I should be checked under the user's experimental conditions (3). It is convenient to prepare 2 mM standard solutions which are diluted to about 0.35 mM for analysis. The blanks consist of 0.5 cc. aliquots of the same sample of solvent.

Use of the color yield of 0.27 for proline at 440 mμ is the same as multiplying the leucine equivalents by 3.7, as previously described (3). For publication, the proline curve has been corrected, whereas the other peaks have been left in terms of leucine equivalents.

In most instances, the choice of fractions to be included in the integration of a given amino acid peak is evident from the graph of the results. In those cases in which the valley between two peaks does not fall to the base-line, one-half of the quantity of amino acid represented by the low point of the valley is assigned to each peak. This procedure has given satisfactory integrations when the valley is less than half the height of the smaller of the two peaks. In the present experiments, no pairs of peaks have been encountered which required an attempt to apply the method of calculation for overlapping components used in the case of tyrosine and valine in the butyl-benzyl alcohol solvent (2).

In the experiment shown in Fig. 2, proline overlaps glutamic acid. The entire glutamic acid curve is read at 570 mμ, and the integration subsequently corrected for the contribution of proline, which has a color yield of only 0.05 (relative to leucine as 1.00) at this wave-length (*cf.* (3)).

Quantitative Analysis of Synthetic Mixtures—The results obtained by the integration of the curves in chromatograms of the type shown in Fig. 1 are summarized in Table II. The synthetic mixture of amino acids corresponded in composition to an acid hydrolysate of bovine serum albumin. Cysteine was omitted since, as will be shown later (4), it was found not to

be present in protein hydrolysates that had been repeatedly evaporated to dryness in order to remove excess HCl.

The separation of phenylalanine from leucine plus isoleucine is not sufficient to permit fully reliable division of the peaks. Since leucine and isoleucine are usually present in by far the larger quantity, the percentage recovery may be fairly accurate for these two amino acids. The phenylalanine values, although more variable, are frequently accurate to ±5 per cent. If the column is loaded more heavily, however, as is sometimes

TABLE II

Recovery of Amino Acids from Known Mixture Containing Eighteen Components

Solvents, 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1 N HCl followed, after the emergence of aspartic acid, by 2:1 *n*-propyl alcohol-0.5 N HCl (*cf.* Fig. 1).

Constituent	Amount present	Per cent recovery			
		Chromatogram 456	Chromatogram 457	Chromatogram 520	Average
	mg.				
Leucine-isoleucine	0.364	99.4	99.5	101.5	100.3
Phenylalanine	0.165	94.8	96.1	94.8	95.2
Valine-methionine-tyrosine	0.354	99.6	101.0	100.1	100.2
Proline	0.136	99.7	97.8	100.0	99.2
Glutamic acid*-alanine	0.515	95.2	94.6	96.8	95.5
Threonine	0.201	97.5	101.0	102.0	100.2
Aspartic acid*	0.267	93.5	94.1	94.7	94.1
Serine	0.118	100.0	99.8	101.2	100.3
Glycine	0.051	99.1	100.5	101.0	100.2
Ammonia	0.024	102.0	99.5	104.5	102.0
Arginine	0.143	97.7	102.8	105.0	101.8
Lysine	0.302	96.3	103.0	99.5	99.6
Histidine	0.094	99.7	104.6	97.4	100.6
Cystine	0.133	89.5	102.7	101.5	97.9
All constituents	2.867	97.3	99.3	99.6	98.7

* When the value for glutamic acid is corrected for the 7 per cent low recovery due to esterification, the recoveries for glutamic acid plus alanine become 100.2, 99.7, and 101.7 per cent. The aspartic acid recoveries, which run 6 per cent low, may be similarly corrected to yield the figures 99.4, 99.9, and 100.8 per cent. The total recoveries, on this basis, become 98.6, 100.8, and 101.0 per cent.

desirable in order to attain higher accuracy in the analysis for the basic amino acids, the resolution of leucine plus isoleucine and phenylalanine becomes less satisfactory than that indicated by Table II. Valine, methionine, and tyrosine are integrated as a group. On an unknown solution, the principal calculation of value for these combined peaks is the estimation of the total amino nitrogen in leucine equivalents.

Proline and threonine emerge as well defined peaks before the solvent shift and are recovered quantitatively. Adjacent to them, however, are the peaks for glutamic acid plus alanine and aspartic acid for which, it will be noted, the recoveries are low. It has been found that the yields of glutamic and aspartic acids are low in this solvent as a result of esterification. With unknown mixtures, the aspartic acid values obtained by integration are divided by 0.94 to give corrected figures.

The procedure which has been outlined for the establishment of the blank after the solvent shift permits quantitative recoveries to be obtained for the peaks emerging after the change to 2:1 *n*-propyl alcohol-0.5 N HCl.

The results obtained in the separation of glutamic acid and alanine with 2:1:1 *tert*-butyl alcohol-*sec*-butyl alcohol-0.1 N HCl are summarized in

TABLE III

Recovery of Glutamic Acid, Alanine, and Other Amino Acids from Synthetic Mixture

Solvent, 2:1:1 *tert*-butyl alcohol-*sec*-butyl alcohol-0.1 N HCl (*cf.* Fig. 2). The mixture contained eighteen components (*cf.* Table II).

Constituent	Amount present	Per cent recovery			
		Chromatogram 474	Chromatogram 543	Chromatogram 481	Average
	mg.				
Leucine-isoleucine	0.373	99.0	100.8		99.9
Phenylalanine	0.169	101.6	103.6		102.6
Valine-methionine-tyrosine	0.363	100.6	104.4		102.5
Glutamic acid	0.426	96.3	97.8	100.2	98.1
Alanine	0.102	97.3	101.3	97.5	98.7

Table III. Esterification of glutamic acid is negligible in this solvent mixture, as evidenced by the essentially quantitative recovery of the amino acid. The chromatogram also provides an alternative determination of phenylalanine, which is well separated in this case. If methionine is absent, the column can yield quantitative values for tyrosine and valine. In most instances, the column has been used only for the separation of glutamic acid and alanine. The valley does not fall to the base-line, and it sometimes is necessary to reduce the load on the column in order to obtain adequate resolution.

Accuracy of Chromatographic Analysis—In general, the chromatographic procedure on starch columns is capable of yielding recoveries of 100 ± 3 per cent. The average recoveries for the components of the synthetic mixture used in the chromatograms summarized in Tables II and III are well within this range. The deviations which do occur appear to be random

and cancel out, in part, in the calculation of the total recovery for the sum of the amino acids, which is almost invariably accurate to ± 1 per cent. In any given experiment, however, a number of factors operate to reduce the accuracy of the analysis for one or more of the constituents. The amount of a given amino acid present is the principal variable. When a mixture contains ten or twenty components, it is probable that a loading for the column which is optimum for some will not be the most favorable for all of the amino acids. When the optical density readings on the peak points of a curve are as low as 0.20, a variation of 0.01 unit in the blank can cause an error of 10 per cent in the integration. Some of the peaks integrated for Table II fall into this category. The accuracy of the recoveries indicates that, in practice, the averaging of a series of blanks usually establishes the base-line to considerably better than 0.01 optical density unit. But the determination is on a sounder basis if the load on the column can be increased to give a peak reading of 0.50 to 1.00 optical density unit. An increase in the total load, however, as has already been mentioned, can have an adverse effect upon the degree of resolution obtained in the case of components present in relatively large amounts. An attempt to obtain an adequate picture of the composition of a mixture in a single chromatogram will usually require a compromise on the question of the optimum load for the column. If the emphasis is on the determination of only a few specific constituents of the mixture, the load can be adjusted to give maximum accuracy for these amino acids. In the case of low peaks, it should also be possible to obtain increased accuracy by using 4 times the present sample size on a column 2 cm. in diameter, if 2 cc. effluent fractions are collected and concentrated to 0.5 cc. before analysis.

DISCUSSION

Identification of Amino Acid Peaks—A discussion has already been given (2) of the general measures which can be taken to assist in the identification of a peak on an effluent concentration curve. The problems associated with the interpretation of the results obtained with unknown mixtures were enumerated for the butyl-benzyl alcohol experiments (2) and apply with added emphasis to the present curves. The positions of the peaks shown in Fig. 1, together with the points of emergence of a number of additional amino acids[6] and related compounds, have been summarized in Table IV. The absolute value for the position of a peak is not as useful an aid in identification as it was in the case of simpler chromatograms. As in the earlier experiments, the relative positions of the peaks are highly reproducible. The same general pattern has been obtained routinely on

[6] We are indebted to Dr. A. Hiller and Dr. D. D. Van Slyke for a sample of hydroxylysine, to Dr. H. T. Clarke for a sample of cysteic acid, and to Dr. H. Borsook for a sample of α-aminoadipic acid.

both synthetic mixtures and protein hydrolysates. In a given experiment, however, all the peaks may emerge somewhat faster or slower than indicated by Table IV. Shifts of as much as 10 per cent have been obtained. These deviations can result from a number of causes, among which may be mentioned small variations in the amount of starch introduced during the pouring of the column, slight differences in the composition of the solvent mixtures, and errors in the adjustment of the size of the fractions collected.

TABLE IV

Order of Emergence of Amino Acids and Related Compounds

Determined on columns 0.9 × 30 cm. prepared from 13.4 gm. of starch (anhydrous), developed with 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1 N HCl and shifted to 2:1 *n*-propyl alcohol-0.5 N HCl at 83 cc.

Compound (*cf.* Fig. 1)	Position of peak	Additional compounds	Position of peak
	cc.		*cc.*
Leucine-isoleucine	13.5	Diiodotyrosine	12.5
Phenylalanine	16.5	Tryptophan	18
Valine	24	α-Amino-*n*-butyric acid	38
Methionine	26	α-Aminoadipic acid	41
Tyrosine	28	Cysteic acid	64
Proline	52	Taurine	74
Glutamic acid-alanine	59	Hydroxyproline	80
Threonine	75	Sarcosine	84
Aspartic acid	82	Citrulline	98.5
Serine	100	Ethanolamine	102
Glycine	106	Asparagine	121
Ammonia	117	Glucosamine	126
Arginine	136	Histamine	160
Lysine	149	Ornithine	176
Histidine	163	Hydroxylysine	180
Cystine	179		

The exact point of the solvent shift, of course, affects the positions of the peaks after aspartic acid. These variations mean that, in a given chromatogram, a peak emerging at 163 cc., for example, cannot be stated to occur at the histidine position, unless it has been placed either by reference to the sequence of the other peaks from the sample or by observance of the rise of the peak at this position after the addition of known histidine.

Considerable variations have also been observed in the absolute positions of the peaks in Fig. 2. The deviations are believed to result from variations in the moisture content of the samples of *tert*-butyl alcohol from which the solvent has been prepared. If proline is present, its characteristic color in the ninhydrin reaction serves to identify the beginning of the glutamic acid peak.

The relative positions of the peaks are fully reproducible only if the column has not been overloaded. The amounts of each amino acid used for Fig. 1 are low enough so that the column is capable of yielding fairly symmetrical effluent curves. As the load of a given component is increased, a point is reached at which the peak in question begins to show a steep front, indicative of a non-linear isotherm. The tail portion of the peak is identical in position and slope with the right-hand half of the peak in Fig. 1, but the increased load will have advanced the point of maximum concentration 1 to 3 cc. ahead of its former position. If this trend is extended by increasing the load to 10 to 20 times the present level, the position of the advancing front is markedly moved ahead. In general, a 2-fold increase over the amounts given in Table II does not lead to significant distortion of the peaks, but, as already mentioned, the degree of increase in the load which is tolerable will depend upon the objectives of the given experiment.

The second column of Table IV gives the positions of some amino acids and related compounds not covered by Fig. 1. As the number of possible components in a mixture is increased, the problems of identification are multiplied, and no general solution can be offered. By the use of additional solvent mixtures, a number of the overlaps in Table IV may be resolved. Diiodotyrosine emerges with leucine and isoleucine, but can be differentiated on a butyl-benzyl alcohol chromatogram (2). Tryptophan coincides with phenylalanine in the solvent mixture referred to in Table IV, but can be determined with 0.1 N aqueous HCl (*cf.* Fig. 3). In acid hydrolysates of proteins the problem seldom arises, since tryptophan is usually decomposed during the hydrolytic process (2). Either α-amino-*n*-butyric acid or α-aminoadipic acid, if present, would appear as a new peak midway in the valley between tyrosine and proline. Cysteic acid has been found to give a clearly defined peak on the right side of the curve for glutamic acid plus alanine. Taurine is indistinguishable from threonine in this solvent, but moves ahead of glutamic acid in 2:1:1 *tert*-butyl alcohol-*sec*-butyl alcohol-0.1 N HCl. Hydroxyproline travels at a rate similar to that of aspartic acid. Although hydroxyproline cannot be determined in this solvent mixture, its color yield is only 0.03 at 570 mμ, and unless present in unusually large quantities, it will not interfere with the estimation of aspartic acid. Citrulline and ethanolamine are slightly to the left and the right, respectively, of serine. The presence of either of these substances will be manifested by a broadening of the peak in the serine position. Glucosamine, if present, would appear as a peak midway between ammonia and arginine. Ornithine and hydroxylysine both coincide with cystine. With protein hydrolysates, therefore, the maximum possible amount of cystine present should be calculated from the total sulfur minus the methionine sulfur. If the amount of ninhydrin color in the cystine position ex-

ceeds that allowed by the calculation, the presence of additional components in the cystine range is indicated.

The fact that D-, L-, and DL-amino acids travel at the same rates on starch columns (2) has been checked in the present experiments with the L and DL forms of proline, glutamic acid, alanine, threonine, aspartic acid, and serine.

Behavior of Cysteine—When a freshly prepared solution of cysteine hydrochloride is added to a column with 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1 N HCl as the solvent, the amino acid is gradually oxidized to cystine and no cysteine peak is obtained in the effluent. No ninhydrin-

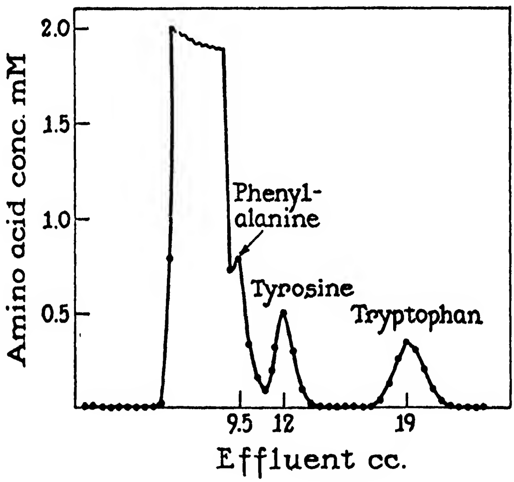


FIG. 3. Separation of tryptophan, with 0.1 N HCl as solvent, from a synthetic mixture containing eighteen components.

positive material emerges from the column until after the shift of solvent to 2:1 *n*-propyl alcohol-0.5 N HCl. In the range of arginine a long flat zone begins and continues up to the position of cystine, where a definite peak occurs. The absorption maximum of the material in this broad zone is at 570 mμ, whereas the absorption maximum of cysteine is at 470 mμ (3). The acidity of the initial solvent is thus insufficient to maintain cysteine in the reduced state. When this amino acid is allowed to stand in the 1:2:1 solvent at atmospheric pressure, about 45 per cent of the cysteine is oxidized in 24 hours. The rate of oxidation on the column is probably accelerated by the increased amount of air in the solvent which enters the column under 15 cm. pressure.

Cysteine, if present in a sample of amino acids applied to the column, would interfere with the determinations of the basic amino acids. In the

chromatographic work with protein hydrolysates, however, the presence of cysteine has not, thus far, been detected (*cf.* (4)).

Cysteine is fairly stable in the more strongly acid solvent, 2:1 *n*-propyl alcohol-0.5 N HCl. If the column is run from the beginning with this solvent mixture, a cysteine peak (absorption maximum 470 mμ) is obtained near the position of threonine (*cf.* Fig. 4).

Separation of Tryptophan—The behavior of tryptophan on a column run with aqueous 0.1 N HCl has been referred to previously (1, 2). The column for this purpose is poured in butyl alcohol and washed with 1:1 *n*-propyl alcohol-0.5 N HCl as usual before being shifted to 0.1 N HCl. The curve obtained with the synthetic bovine serum albumin mixture to which tryptophan had been added is given in Fig. 3. The first peak contains most of the components of the mixture. Only the aromatic amino acids are appreciably retarded, and tryptophan emerges as a completely separated peak. The color yield of tryptophan in the neutralized 0.5 cc. samples has been 0.72 (3) and the recoveries from the chromatogram have been 100 ± 3 per cent.

Esterification in Acidic Solvents—The fact that the amino acids should not be allowed to stand in an acidic alcoholic solvent before the sample is placed on the column has been noted previously (2). Aspartic and glutamic acids are the only amino acids which have shown measurable esterification on the starch column during the course of the present experiments. The degree of ester formation is a function of the amount of water in the solvent mixture, the HCl concentration, the nature of the alcohols, and the time of contact. In the chromatograms with 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1 N HCl the recoveries of aspartic and glutamic acids have been 6 and 7 per cent low, respectively. The percentage loss is independent of the amounts of the amino acids present. If the column is developed from the beginning with 2:1 *n*-propyl alcohol-0.5 N HCl (*cf.* Fig. 4), the losses of these two amino acids are 10 and 20 per cent. On a column developed with *n*-butyl alcohol-17 per cent 0.57 N HCl (2), the recoveries are low by 20 and 30 per cent.

If the synthetic mixture of amino acids is allowed to stand for 1 week in the 1:2:1 solvent before the sample is placed on the column, two small additional peaks appear ahead of leucine plus isoleucine in Fig. 1. The yield of glutamic acid is about 20 per cent low and that of aspartic acid about 10 per cent low. All other components are quantitatively recovered. The small amount of esterification which occurs during the usual chromatographic experiment is not manifest in any way other than in the reduction of the yields of aspartic and glutamic acids. The esters, as they are continuously formed, move fairly rapidly through the column and doubtless contribute some ninhydrin color to all the effluent fractions preceding the

glutamic and aspartic acid peaks. The quantity of ester is so small, however, and is distributed over so many fractions that the increase in ninhydrin color for any given fraction is almost imperceptible.

It has already been noted that in the mixture of secondary and tertiary alcohols used for the separation of glutamic acid and alanine (Fig. 2) esterification appears to be negligible.

Studies on Other Solvent Mixtures—In the chromatographic separation of the faster moving amino acids described in the previous communication (2), neutral water-immiscible organic solvents such as *n*-butyl alcohol and benzyl alcohol were used with columns 30 cm. in height. In order to elute some of the slower moving amino acids from such columns, inconveniently large effluent volumes are required. As the concentrations in the effluent become more dilute, the analytical accuracy is decreased. By the use of these same solvents with shorter columns (7.5 cm. in height), proline, alanine, and threonine can be eluted satisfactorily (1). The number of effective plates in a column, however, or the potential resolving power of the chromatogram is proportional to its length, and consequently it is preferable to use the longest column compatible with convenient laboratory operation. In order to attain satisfactory rates of travel for the slower moving amino acids on columns 30 cm. in length, a variety of solvent mixtures have been studied.

Neutral *n*-propyl alcohol-water mixtures were investigated on starch columns poured in butanol, washed to constant blank with the neutral solvent, and treated with 8-hydroxyquinoline (2). With 2:1 *n*-propyl alcohol-water, a curve was obtained which was similar to the first portion of Fig. 1, except that glutamic acid and aspartic acid were not present as discrete peaks but were spread out in a long low plateau extending from 60 to 100 cc. of the effluent. The other amino acid peaks emerged somewhat ahead of their positions in Fig. 1, threonine being at 51 cc. The last peak, which emerged at 71 cc., was composed of both serine and glycine. There also appeared, between proline and alanine, a large artifact peak which proved to result from ninhydrin-positive material eluted from the starch by the HCl in the amino acid sample. It was found that a small amount of either HCl or NaCl, when added to the top of the column, was capable of liberating material containing amino nitrogen, which moved down the column as a discrete zone and emerged as an irregular peak just ahead of the alanine position. The 2:1 *n*-propyl alcohol-water experiment provided a possible determination of proline, alanine, and threonine. The presence of the artifact peak and the unsatisfactory behavior of the acidic amino acids were marked disadvantages.

Glutamic acid and aspartic acid were obtained as normally sharp peaks in the alanine-threonine range when 0.25 N acetic acid was substituted

for water in the 2:1 mixture with n-propyl alcohol. The artifact peak was still present, however, and there was overlapping of the components. In an attempt to eliminate the artifact, the starch column was treated with HCl and propyl alcohol, as described in the experimental section, until all ninhydrin-positive material had been eluted. The solvent was then changed to 2:1 n-propyl alcohol-water. When an amino acid mixture containing no HCl or NaCl was added to the column, the amino acid peaks were markedly retarded by the acid-washed starch. As an alternative procedure, a column was cleaned until ninhydrin-negative by using 2:1 n-propyl alcohol-0.1 N NaCl. The column was washed free of chloride ion with 2:1 n-propyl alcohol-water and the amino acid sample was added as usual. With the NaCl-washed starch the neutral amino acid peaks were sharp and there was no artifact zone. The peaks of the acidic amino acids, however, although they appeared in the proper range, were markedly broadened. As a result the curve was similar to Fig. 1, except that alanine, glutamic acid, threonine, and aspartic acid emerged as a group. Serine and glycine gave overlapping peaks at 67 and 71 cc. The inclusion of 0.5 N acetic acid or 0.5 N pyridine in the solvent did not improve the resolution in the acidic amino acid range. If acid-washed starch was suspended briefly in dilute NaOH and washed free of alkali, a product was obtained which behaved similarly to NaCl-washed starch.

Thus, the acidic amino acids have not yielded fully satisfactory results on starch columns developed with neutral unbuffered solvents. In addition, the properties of starch are such that both unwashed and NaCl-washed samples have a strong affinity for the basic amino acids. Even when only water is used as the solvent, the basic amino acids travel extremely slowly. Although the characteristics of neutral columns have thus not proved favorable for analytical work, it is possible that they may be useful in certain cases for preparative experiments. The effluent contains a minimum of carbohydrate impurities, whereas the effluent from columns run with acidic solvents is ninhydrin-negative but not carbohydrate-free. Although the columns prepared with acidic solvents retain their efficiency over periods of several weeks, starch is not fully stable under these conditions and some carbohydrate material is continuously passing into the effluent. The separation of amino nitrogen-containing constituents from carbohydrates in the effluent does not present major difficulties in some cases, but further work is required to facilitate the isolation of components from the effluent of columns run with acidic solvents.

In early experiments, attempts were made to achieve satisfactory rates of travel of amino acids on the column simply by varying the water content of propanol-water mixtures. It was found, however, that, although the

amino acids emerged at greater effluent volumes as the amount of water in the solvent was decreased, this retardation was accompanied by a broadening and flattening of the peaks when the water content was reduced below about 30 per cent. Thus, 3:1 *n*-propyl alcohol-water gives a curve in which a peak emerging at a given effluent volume is slightly lower and broader than its counterpart in a 2:1 solvent. If the water content is reduced from 25 to 20 per cent, a comparison of the peaks emerging at the same effluent positions shows those in the 4:1 solvent to be about halved in height and doubled in width. A further reduction in the amount of water, to 12 per cent, causes the amino acids to emerge at a fairly steady

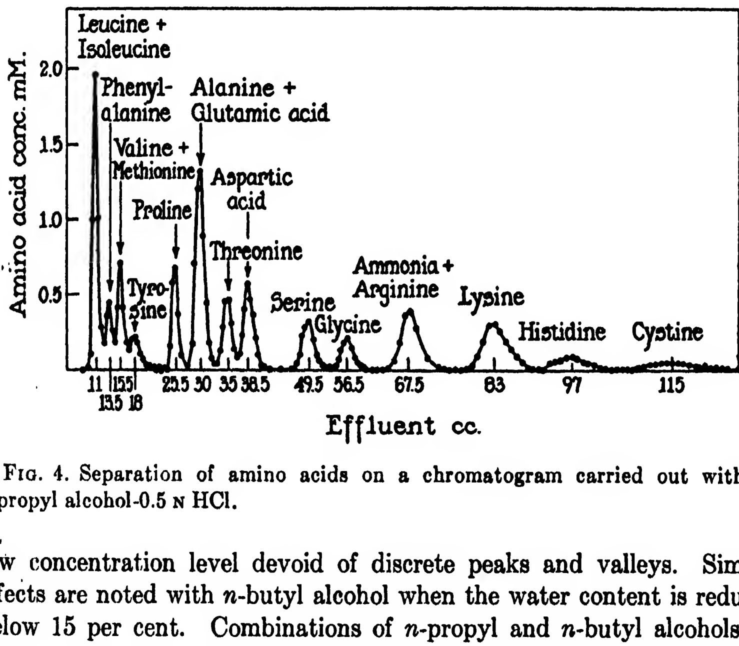


FIG. 4. Separation of amino acids on a chromatogram carried out with 2:1 *n*-propyl alcohol-0.5 N HCl.

low concentration level devoid of discrete peaks and valleys. Similar effects are noted with *n*-butyl alcohol when the water content is reduced below 15 per cent. Combinations of *n*-propyl and *n*-butyl alcohols, as used in the 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1 N HCl solvent, permit mixtures to be employed which have water contents intermediate between 15 and 30 per cent without there being manifest any undesirable broadening of the peaks.

It may prove desirable for some purposes to run the column from the beginning with 2:1 *n*-propyl alcohol-0.5 N HCl, instead of employing a solvent of lower water and HCl content for the first part of the curve. The results of such an experiment are shown in Fig. 4. The resolution of the faster moving amino acids is less satisfactory than in Fig. 1. The losses of glutamic and aspartic acids as a result of esterification are greater, as already noted. The fact that ammonia and arginine emerge together

is a disadvantage. Nevertheless, the solvent may have some utility for screening work. A general picture of the composition of a mixture of amino acids is obtained in a 4 day experiment, instead of the 7 days required to obtain the results shown in Fig. 1.

Many experiments with acidic solvents other than the ones already described were carried out in an attempt to increase the resolution of the amino acids in the proline-aspartic acid range. Usually a preliminary experiment was performed with the synthetic serum albumin mixture. Inspection and integration of the curves were frequently sufficient to eliminate a given solvent from further consideration. Some of the solvents were investigated in greater detail with simpler mixtures of amino acids. The only combination found which would completely separate glutamic acid and alanine was 3:1 *tert*-butyl alcohol-0.1 N HCl. Because of its viscosity, this solvent gives excessively slow flow rates on the starch columns and has not been used routinely. The incorporation of 25 per cent *sec*-butyl alcohol in the mixture has given a satisfactory flow rate and reasonably good separation of the two amino acids. Various other mixtures of 0.1 N HCl with *sec*-butyl alcohol, *tert*-butyl alcohol, isobutyl alcohol, isopropyl alcohol, methyl cellosolve, and butyl cellosolve were tried. The mixtures did not offer any general advantages over the solvents referred to in Figs. 1 and 2.

In the present experiments, emphasis has been focused on solvents containing no non-volatile acids or salts which would tend to complicate the possible isolation of constituents from the effluent. A few chromatograms have been run with buffered solutions and with non-volatile acids. In 2:1 *n*-propyl alcohol-0.5 N H_3PO_4, the results were fairly similar to those shown in Fig. 4. In 2:1 *n*-propyl alcohol-0.5 N trichloroacetic acid, the basic amino acid peaks were advanced to positions on top of the components in the alanine-glycine range. No advantages in the proline-aspartic acid range were afforded by the use of 2:1 *n*-propyl alcohol-0.5 N monochloroacetic acid. With 2:1 *n*-propyl alcohol-0.2 N citric acid the peaks were markedly broadened and resolution was inferior.

When buffered solutions are used on starch columns, sharp peaks are obtained with both the acidic and basic amino acids. In 2:1 *n*-propyl alcohol-0.2 M citrate buffer, pH 5, the curve was similar to that in Fig. 1, except that glutamic acid and aspartic acid were shifted to the right. Glutamic acid emerged at a position on top of serine and glycine and was followed by the aspartic acid peak. The chromatogram was not continued to cover the basic amino acid range. When a citrate buffer of pH 4 was used, the basic amino acids were moved up to give an overlapping zone with glycine, serine, ammonia, and the acidic amino acids. In 3:2 *n*-propyl alcohol-0.08 M citrate buffer, pH 8, the relative rates of travel of the

basic amino acids were further increased to give a heavily bunched group in the center section of the curve. Solvents that are much more alkaline than pH 8 cannot be used with starch. With 0.1 N NaOH, the starch at the top of the column swells and gelatinizes in the presence of the strong alkali.

Thus, both organic acids and the citrate buffers of pH 4 and 8 increase the rates of travel of the basic amino acids relative to the monoamino acids, thereby increasing the probability of overlaps in the chromatogram. The use of HCl possesses the advantage that minimum rates of travel for the basic amino acids are obtained, placing them in a region to the right of glycine.

SUMMARY

Chromatographic fractionation of amino acids on starch columns has been extended to include most of the common constituents of protein hydrolysates. The principal solvent mixture which has been used is 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1 N HCl followed, after the emergence of aspartic acid, by 2:1 *n*-propyl alcohol-0.5 N HCl. In experiments with synthetic mixtures containing seventeen amino acids and ammonia, this combination of solvents yields in a single chromatogram a curve which includes all the components, with a few overlaps. For analytical work, about 2.5 mg. of the amino acid mixture are required per chromatogram. Integration of the curves has given quantitative recoveries for proline, threonine, aspartic acid, serine, glycine, ammonia, arginine, lysine, histidine, and cystine. Glutamic acid and alanine emerge together but can be resolved in a separate chromatogram with 2:1:1 *tert*-butyl alcohol-*sec*-butyl alcohol-0.1 N HCl. The six most rapidly moving components are partially resolved and have been separated, as previously reported, on columns run with 1:1:0.288 *n*-butyl alcohol-benzyl alcohol-water for the determination of phenylalanine, leucine, isoleucine, methionine, tyrosine, and valine. Thus, by the use of three starch columns it is possible to separate from one another all the eighteen components.

The average recoveries in duplicate or triplicate determinations have been 100 ± 3 per cent. The positions of emergence of some of the less commonly occurring amino acids and related compounds have been determined. Tryptophan, although not usually present in acid hydrolysates, presents a special case and can be determined on a column developed with aqueous 0.1 N HCl. If desired, a variety of other solvents, including neutral, acidic, and buffered solvent mixtures, can be used satisfactorily with starch columns.

The authors wish to acknowledge the assistance of Miss Enid Mellquist and Mr. H. R. Richter in the performance of this work.

BIBLIOGRAPHY

1. Moore, S., and Stein, W. H., *Ann. New York Acad. Sc.*, **49**, 265 (1948).
2. Stein, W. H., and Moore, S., *J. Biol. Chem.*, **176**, 337 (1948).
3. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **176**, 367 (1948).
4. Stein, W. H., and Moore, S., *J. Biol. Chem.*, **178**, 79 (1949).
5. Tiselius, A., in Anson, M. L., and Edsall, J. T., Advances in protein chemistry, New York, **3** (1947).

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, March, 1949,
Vol. 178, No. 1, pp. 79–91]

AMINO ACID COMPOSITION OF β-LACTOGLOBULIN AND BOVINE SERUM ALBUMIN

BY WILLIAM H. STEIN AND STANFORD MOORE

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, October 8, 1948)

The experiments described in this communication are concerned with the use of starch columns (1–3) for the chromatographic determination of the amino acid composition of protein hydrolysates. The techniques outlined in the preceding paper (3) have been employed to analyze hydrolysates of β-lactoglobulin and bovine serum albumin. In conjunction with the results of earlier chromatograms run with the butyl-benzyl alcohol solvent system (1), the analyses appear to have accounted for all the amino acids present in acid hydrolysates of these two proteins. Since numerous amino acid determinations have already been reported for both β-lactoglobulin and bovine serum albumin, an opportunity is afforded for comparison of the chromatographic data with the results obtained by other methods.

The experimental procedure used for the chromatographic analyses was identical with that employed in the preceding studies with synthetic mixtures of amino acids (3). The sample of β-lactoglobulin used in this work was prepared by Dr. G. Haugaard according to the method of Palmer (4, 5), and was one of the samples recently analyzed by Brand and coworkers (6). The bovine serum albumin, prepared according to the method of Cohn *et al.* (7), was obtained through the kind cooperation of Dr. Erwin Brand, and was the same preparation (Armour, Lot 18) analyzed in his laboratory. Hydrolysis was performed in the manner already outlined (1), with 200 volumes of 6 N HCl twice distilled in glass. For convenience in manipulation, 250 to 500 mg. of protein were hydrolyzed. Since the chromatographic analyses require only 2.5 mg. per experiment, the procedure for hydrolysis and for addition of the sample to the column can be scaled down, if desired, to permit the series of chromatograms to be completed with 25 to 50 mg. of protein.

Analyses of Hydrolysates of β-Lactoglobulin

A hydrolysate of β-lactoglobulin was chromatographed with 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1 N HCl and 2:1 *n*-propyl alcohol-0.5 N HCl as solvents. The resulting effluent concentration curve, given in Fig. 1, is similar in all respects to the curve obtained in experiments with a synthetic mixture containing seventeen amino acids and ammonium chloride (3). The general pattern and the approximate positions of the peaks

are the same in the two curves. There are no peaks in Fig. 1 which cannot be ascribed to the common amino acids. No evidence has been obtained, therefore, for the existence in β-lactoglobulin hydrolysates of unsuspected amino nitrogen-containing constituents. In order to confirm the positions assigned to the peaks in Fig. 1, threonine, serine, and histidine were added to the hydrolysate. The designated peaks rose without loss of symmetry. Since β-lactoglobulin is a protein, the composition of which has already been explored rather fully by a variety of methods, the identification of the peaks in the chromatogram can be made with a relatively high degree of certainty (1, 3). Characterization of the components of the peaks by

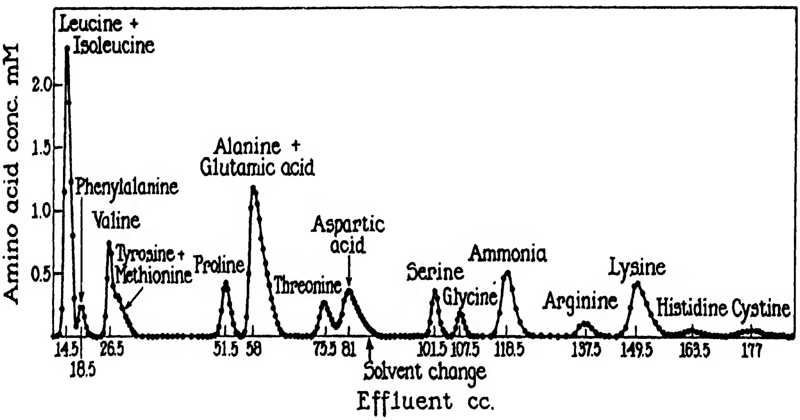


FIG. 1. Chromatographic fractionation of a hydrolysate of β-lactoglobulin. Solvents, 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1 N HCl and 2:1 *n*-propyl alcohol-0.5 N HCl. Column dimensions, 0.9 × 30 cm. Sample, an amount of hydrolysate corresponding to about 2.5 mg. of protein.

isolation of the amino acids from the effluent has not been attempted in these analytical experiments.

Integration of Effluent Curves—The quantitative data yielded by the experiment shown in Fig. 1, and replicates thereof, are given in Table I. The total nitrogen recoveries of 98.9 and 99.7 per cent indicate that the chromatogram has almost completely accounted for the nitrogenous components of the hydrolysate. There are several approximations involved, however, in this calculation. As already mentioned (3), the peak for valine plus methionine plus tyrosine yields only an approximate value for the per cent of the total nitrogen of the protein attributable to these combined amino acids. The peak is integrated by using a color yield of 1.00, whereas the respective color yields for the individual components are 1.01, 1.00, and 0.86. The recoveries obtained for the first six fast moving com-

ponents in Fig. 1 are useful in the preparation of a nitrogen balance sheet for the chromatogram, but are replaced by the results from a butyl-benzyl alcohol column in later calculations. The glutamic acid plus alanine integration also yields a slightly low figure, since a correction for losses of glutamic acid as a result of esterification is not included. Tryptophan is not present in the acid hydrolysate, as evidenced by the absence of ninhydrin-positive material in the valley between phenylalanine and leucine

TABLE I

Chromatographic Analyses of Hydrolysates of β-Lactoglobulin

Solvents, 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1 N HCl and 2:1 *n*-propyl alcohol-0.5 N HCl (*cf.* Fig. 1).

Constituent	Chromatogram 472		Chromatogram 491		Chromatogram 486*
	Gm. amino acid per 100 gm. protein	N as per cent of protein N	Gm. amino acid per 100 gm. protein	N as per cent of protein N	Gm. amino acid per 100 gm. protein
Leucine-isoleucine	22.6	15.47	22.4	15.34	
Phenylalanine	3.85	2.09	3.89	2.12	
Valine-methionine-tyrosine		8.38 *ca.*		8.30 *ca.*	
Proline	5.29	4.13	5.16	4.03	4.96
Glutamic acid-alanine		18.60 *ca.*		19.35 *ca.*	
Threonine	4.85	3.65	4.75	3.58	4.42
Aspartic acid	11.46	7.74	11.64	7.86	11.47
Serine	3.56	3.04	3.76	3.21	3.37
Glycine	1.35	1.62	1.48	1.77	1.34
Ammonia	1.44	7.61	1.47	7.76	1.45
Arginine	3.07	6.34	2.74	5.66	2.91
Lysine	12.25	15.05	12.70	15.60	12.80
Histidine	1.49	2.59	1.61	2.80	1.78
Cystine	3.52	2.63	3.15	2.35	3.09
Total nitrogen recovery		98.9		99.7	

* A 40 cc. fore fraction was collected and the column placed on the fraction collector before the emergence of the proline peak.

in the earlier butyl-benzyl alcohol chromatograms (1). Since tryptophan represents 1.71 per cent of the protein nitrogen, as determined by Brand and coworkers (6) who published the first approximately complete analysis of β-lactoglobulin, the theoretical recovery for Table I should be 98.3 per cent. For some purposes, such as comparative studies on different protein preparations, the type of data afforded by Table I may be adequate without the performance of additional chromatograms. Quantitative figures are obtained for ten of the individual components, and an approximate total nitrogen recovery may be derived from the single chromatogram.

The hydrolysates have given a negative nitroprusside test. In addition, there was no evidence of interference from cysteine in the valleys on either side of the histidine peak. Apparently any cysteine originally present is oxidized during the preparation of the hydrolysate for analysis.

In the present experiments the data from Table I have been combined with the results from additional columns to give a more complete picture of the composition of the hydrolysate. Glutamic acid and alanine have been determined with 2:1:1 *tert*-butyl alcohol-*sec*-butyl alcohol-0.1 N HCl (3). A fore fraction was collected and the chromatogram placed on the fraction collector prior to the emergence of proline. Duplicate determinations gave values of 19.30 and 18.85 per cent for glutamic acid, and 7.10 and 7.08 per cent for alanine. The results obtained in the experiments with *n*-butyl alcohol-benzyl alcohol columns have already been reported (1).

Composition of β-Lactoglobulin—The amino acid composition of β-lactoglobulin indicated in Table II is based in major part upon the analytical results from the three types of chromatograms discussed above. From the standpoint of protein composition, data of the kind given in Table II are always subject to uncertainties arising from possible decomposition of various amino acids during hydrolysis. Significant amounts of threonine and serine, for example, are known to decompose during acid hydrolysis, with the formation of ammonia. In the present work, the values of 4.67 and 3.56 for threonine and serine, obtained from the hydrolysate, have been divided by 0.95 and 0.90, respectively, in accordance with the estimates of Rees (17) for the decomposition of these amino acids during a 20 hour period of acid hydrolysis. Although the hydrolytic conditions employed in the present experiments differ from those used by Rees, the parallelism between his results and the present figures indicates that the same correction factors are probably applicable in both cases. For example, Rees obtained uncorrected values of 4.84 and 3.64 per cent for threonine and serine in hydrolysates of β-lactoglobulin. He found the total ammonia content of the hydrolysate to be 1.49 per cent. The corresponding chromatographic value is 1.45 per cent (Table I).

A maximum possible value for the amide ammonia of the protein can be obtained from the chromatographic data by subtracting from the total ammonia content of the hydrolysate the amount of ammonia estimated to be formed by the decomposition of threonine and serine. The resulting figure, 1.35 per cent of ammonia, is slightly higher than the amide ammonia value of 1.31 per cent as determined by Warner and Cannan (20), Brand *et al.* (6), and by Rees (17).

Essential data in Table II derived from other sources include the photometric value of Brand *et al.* (6) for tryptophan and the sulfur partition results obtained by the same authors. The independent value for me-

TABLE II

Amino Acid Composition of β-Lactoglobulin

The values for phenylalanine, leucine, isoleucine, tyrosine, and valine are from chromatograms carried out with 1:1:0.288 *n*-butyl alcohol-benzyl alcohol-water (1). Glutamic acid and alanine were determined with 2:1:1 *tert*-butyl alcohol-*sec*-butyl alcohol-0.1 N HCl. The remaining chromatographic values are the average figures from Table I. The nitrogen content of the protein was 15.6 per cent, on an ash- and moisture-free basis.

Constituent	Gm. amino acid per 100 gm. protein	Gm. amino acid residue per 100 gm. protein	N as per cent of protein N	Literature values, gm. amino acid per 100 gm. protein
Phenylalanine	3.78	3.37	2.06	3.54 (6), 4.3 (8), 4.2 (9)
Leucine	15.50	13.38	10.61	15.7 (7), 15.4 (10), 15.9 (11)
Isoleucine	5.86	5.05	4.01	8.4 (6), 7.0 (8), 6.1 (12)
Methionine	3.22*	2.84	1.94	
Tyrosine	3.64	3.28	1.81	3.78 (6), 3.6 (13)
Valine	5.62	4.76	4.31	5.8 (6), 5.5 (8), 5.8 (9)
Proline	5.14	4.33	4.01	4.1 (6), 5.5 (9), 4.84 (14)
Glutamic acid	19.08	16.75	11.64	19.5 (6), 19.1 (15), 21.51 (16)
Aspartic "	11.52	9.98	7.80	11.4 (6), 11.2 (15), 9.9 (16)
Alanine	7.09	5.67	7.15	6.2 (6), 6.1 (9), 7.05 (14)
Threonine	4.92†	4.18	3.71	5.85 (6), 5.11 (17)
Serine	3.96†	3.28	3.39	5.0 (6), 4.07 (17)
Glycine	1.39	1.06	1.66	1.4 (6), 1.5 (15), 1.56 (14)
Arginine	2.91	2.61	6.00	2.87 (6), 2.89 (16), 2.91 (18)
Lysine	12.58	11.02	15.45	11.4 (6), 11.4 (15), 10.55 (19)
Histidine	1.63	1.44	2.83	1.58 (6), 1.50 (8), 1.55 (16)
Cystine + cysteine	3.40‡	2.89	2.54	
Tryptophan	1.94§	1.77	1.71	
Amide-NH_3	1.31‖		6.93	1.31 (6), 1.30 (17)
Total		97.7	99.6	

* The value for methionine is that determined by Brand *et al.* (6). An independent check on this figure was not obtained with the butyl-benzyl alcohol chromatograms in this case, since the experiments were carried out before the introduction of thiodiglycol as an antioxidant.

† The average threonine and serine values of 4.67 and 3.56 from Table I have been divided by 0.95 and 0.90, respectively, in accordance with the estimates of Rees (17) for decomposition of these amino acids during hydrolysis.

‡ The cystine + cysteine value is that determined by Brand *et al.* (6). The chromatograms gave an average value of 3.25.

§ The tryptophan value is that determined by Brand *et al.* (6).

‖ The amide-NH_3 value is that determined by Warner and Cannan (20). From the chromatographic data a maximum amide-NH_3 value of 1.35 can be calculated.

thionine is necessary in this case, since the butyl-benzyl alcohol chromatograms were performed before the incorporation of thiodiglycol in the solvent as an antioxidant (1). Brand *et al.* (6) obtained a value of 3.40 per cent

for cysteine plus cystine, which, in conjunction with the methionine content, accounted for 99.6 per cent of the sulfur of the protein. The average chromatographic value in Table I is 3.25 per cent. It should be noted that the hydrolytic conditions employed for the chromatographic experiments were not the same as those recommended as optimum for cysteine and cystine determinations by Brand and Kassell (21). Values for cystine in the present hydrolysates have run from 5 to 10 per cent below the accepted figures for cysteine plus cystine. The quantitative recovery of cystine from synthetic mixtures (3) indicates that the chromatogram reflects the true cystine content of the hydrolysate, and that the values are low as a result of some decomposition of cysteine or cystine during the preparation of the hydrolysate. In the present experiments, an attempt has been made to obtain as much information as possible from an HCl hydrolysate. Adequate chromatographic values for the special case of cystine would require studies on hydrolysates prepared by alternative procedures.

The fact that the calculated cystine sulfur from the chromatogram is slightly less than enough to account, together with methionine, for the total sulfur of the protein leaves no room for the presence of ornithine or hydroxylysine as possible overlaps in the cystine range (3).

The total nitrogen recovery of 99.6 per cent in Table II rests primarily on the chromatographic data. The weight recovery is 97.7 per cent. For many purposes this is a satisfactory balance sheet for the protein. The 2 per cent unaccounted for on a weight basis is a discrepancy which may arise from one or more of several causes. There may be some decomposition of amino acids other than those for which corrections have been made. Some of the amino acid analyses may be in error in such a manner as to compensate on a nitrogen basis but not on a weight basis. There may be amino acid constituents present which have not been determined by the chromatograms. Hydroxyproline, for example, would fall under the aspartic acid peak, and would not be detected. Negative colorimetric tests for this amino acid were obtained by Brand *et al.* (6). Recently, Keston, Udenfriend, and Cannan[1] have demonstrated by their isotopic derivative method, using radioactive *p*-iodophenyl sulfonyl chloride, that hydroxyproline is completely absent (<0.05 per cent) in β-lactoglobulin. It is also possible that the sample of protein contains small amounts of non-nitrogenous constituents or impurities in addition to the ash and moisture. The latter have been corrected for in the assignment of a nitrogen content of 15.6 per cent to the protein. In the present state of accuracy of the chromatographic methods, however, it is not possible to base any conclusions on the observed small difference between the weight and nitrogen recoveries.

[1] Keston, A. S., Udenfriend, S., and Cannan, R. K., personal communication.

Comparison of Chromatographic Results with Other Values—Analytical results obtained by other methods are included in Table II. A more complete summary of literature values has been given by Brand (22). On synthetic mixtures of amino acids the chromatograms have yielded recoveries of 100 ± 3 per cent for individual components (1, 3). The microbiological assays, and some of the other methods, have a potential error at least this large. Thus, for purposes of comparison, conformity to within 5 per cent has been considered as reasonable agreement.

For leucine, tyrosine, valine, arginine, and histidine, the several literature values in Table II are all in agreement with the chromatographic results. Glycine also falls in this group if the variation from 1.39 to 1.56 per cent is considered satisfactory for an amino acid present to so small an extent. The threonine and serine values are in excellent agreement with those of Rees (17) obtained by periodate oxidation, as already mentioned, but are about 20 per cent below the results of Brand *et al.* (6). The glutamic and aspartic acid figures are both checks with the isotope dilution values of Foster (15) and the microbiological results of Brand *et al.* (6). They are not in line with the analyses of Chibnall, Rees, and Williams (16). The isoleucine result has been discussed previously (1) and is in agreement with the most recent microbiological determination (12). For phenylalanine, the two microbiological values (6, 8) are more than 5 per cent above and below the chromatographic value from starch columns. The figure obtained by Tristram (9), who employed chromatography of the acetylated amino acids on silica gel, is also higher than the present phenylalanine determination.

Alanine has previously been a difficult amino acid to determine. The present value is higher than the microbiological figure (6) or that of Tristram (9), but is in agreement with the recent results of Keston, Udenfriend, and Cannan (14), obtained by the isotopic derivative method. The proline content is 20 per cent above the microbiological value of Brand *et al.* (6), but in fairly good agreement with the isotopic derivative data (14).

The value for lysine given in Table II is 10 per cent higher than the microbiological (6) and isotope dilution (15) figures. References to additional lysine determinations, all of which fall between 10.5 and 11.4 per cent, are reviewed by Brand (22). The chromatographic value in this instance may be in error. For bovine serum albumin, discussed later, the chromatographic determination of lysine is in good agreement with the isotope dilution data. In the β-lactoglobulin hydrolysate, there may be a small amount of some component other than lysine traveling in the same range on the column, although none of the additional substances studied to date (3) fall in this category. It is also possible that the chromatographic figure may be correct, if there is some racemization of lysine during hydrolysis. It has been shown previously (3) that the sum of the D

and L isomers in the hydrolysate is determined by the chromatographic method. As usually employed, the microbiological (6), isotope dilution (15), and lysine decarboxylase (23) methods permit the determination of only the L isomer.

A calculation of a minimum molecular weight for the protein from analytical figures has been made by Brand *et al.* (6), by adjusting the molar ratios to integers within 3 per cent. For the amino acids present in the smallest amounts in the protein, the attainment of this degree of accuracy by the chromatographic methods, at least, cannot be assured. For purposes of molecular weight calculations, an absolute accuracy of 5 per cent is about as close a limit as can be placed on the smaller figures in Table II. For example, the deviations from the mean in the triplicate analyses for arginine and histidine in Table I emphasize the need for caution in the mathematical interpretation of the results. The determination of histidine (1.63 per cent) to within 5 per cent of its value is equivalent to determining about 0.1 per cent of the total weight of the protein. This is about the limit of accuracy of the chromatographic procedure. The value of any detailed mathematical treatment of the analytical figures in this case is subject to the further limitation that β-lactoglobulin, although it is a protein which can be prepared in a fairly reproducible form, may not be homogeneous (*cf.* (24, 25)).

Analyses of Hydrolysates of Bovine Serum Albumin

The effluent concentration curve obtained upon chromatography of a hydrolysate of bovine serum albumin is given in Fig. 2. The curve parallels that obtained with the synthetic mixture (3) in all respects. No new peaks are present. When threonine, serine, and histidine were added to a sample of the hydrolysate, the designated peaks rose without loss of symmetry, and the added amino acids were recovered in yields of 105, 99, and 99 per cent, respectively.

Integration of Effluent Curves—The quantitative data obtained from the experiment shown in Fig. 2, and replicates thereof, are given in Table III. As was true in the case of β-lactoglobulin, the nitrogen of the hydrolysate has been almost completely accounted for in each experiment. The tryptophan content of bovine serum albumin, according to the data of Brand *et al.* (26, 22), corresponds to only 0.50 per cent of the total nitrogen of the protein, and the theoretical recovery for Table III is thus 99.5 per cent. The observed recovery is subject to the same approximations which were discussed for β-lactoglobulin.

Duplicate chromatograms with 2:1:1 *tert*-butyl alcohol-*sec*-butyl alcohol-0.1 N HCl gave values of 16.7 and 16.3 per cent for glutamic acid and 6.32 and 6.17 per cent for alanine.

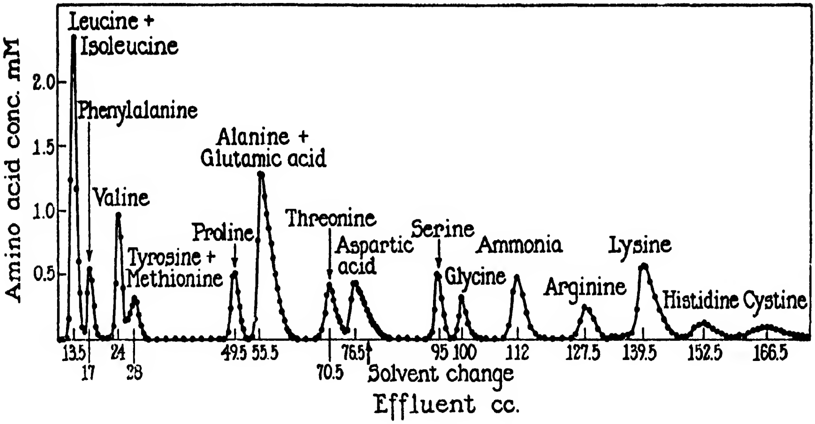


FIG. 2. Chromatographic fractionation of a hydrolysate of bovine serum albumin. Solvents, 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1 N HCl and 2:1 *n*-propyl alcohol-0.5 N HCl. Column dimensions, 0.9 × 30 cm. Sample, an amount of hydrolysate corresponding to about 2.5 mg. of protein.

TABLE III

Chromatographic Analyses of Hydrolysates of Bovine Serum Albumin

Solvents, 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1 N HCl and 2:1 *n*-propyl alcohol-0.5 N HCl (*cf.* Fig. 2).

Constituent	Chromatogram 480		Chromatogram 481		Chromatogram 488	
	Gm. amino acid per 100 gm. protein	N as per cent of protein N	Gm. amino acid per 100 gm. protein	N as per cent of protein N	Gm. amino acid per 100 gm. protein	N as per cent of protein N
Leucine-isoleucine	14.4	9.58	14.75	9.80	14.3	9.51
Phenylalanine	6.32	3.34	6.44	3.40	6.52	3.44
Valine-methionine-tyrosine		7.42 *ca.*		7.37 *ca.*		7.20 *ca.*
Proline	4.65	3.52	4.75	3.60	4.85	3.67
Glutamic acid-alanine		14.90 *ca.*		15.25 *ca.*		15.40 *ca.*
Threonine	5.44	3.98	5.72	4.19	5.50	4.05
Aspartic acid	10.91	7.15	10.86	7.12	10.96	7.19
Serine	3.91	3.24	3.76	3.12	3.77	3.13
Glycine	1.85	2.15	1.75	2.03	1.85	2.15
Ammonia	1.03	5.28	1.08	5.54	1.06	5.44
Arginine	5.96	11.92	6.03	12.07	5.72	11.45
Lysine	12.70	15.15	12.62	15.05	13.15	15.68
Histidine	4.29	7.24	3.67	6.18	4.04	6.82
Cystine	5.83	4.23	5.81	4.22	6.08	4.41
Total nitrogen recovery	.	99.1		98.9		99.5

TABLE IV

Amino Acid Composition of Bovine Serum Albumin

The values for phenylalanine, leucine, isoleucine, tyrosine, and valine are from chromatograms carried out with 1:1:0.288 *n*-butyl alcohol-benzyl alcohol-water containing 0.5 per cent thiodiglycol (1). Glutamic acid and alanine were determined with 2:1:1 *tert*-butyl alcohol-*sec*-butyl alcohol-0.1 N HCl. The remaining chromatographic values are the average figures from Table II. The nitrogen content of the protein was 16.07 per cent, on an ash- and moisture-free basis.

Constituent	Gm. amino acid per 100 gm. protein	Gm. amino acid residue per 100 gm. protein	N as per cent of protein N	Literature values, gm. amino acid per 100 gm. protein
Phenylalanine	6.59	5.87	3.48	6.1 (22), 6.48 (27), 6.05 (28)
Leucine	12.27	10.58	8.17	13.7 (22, 26), 11.8 (27), 13.2 (28)
Isoleucine	2.61	2.25	1.74	2.9 (22), 2.97 (27), 2.7 (28)
Methionine	0.81*	0.71	0.47	0.86 (27), 0.80 (28)
Tyrosine	5.06	4.56	2.44	5.5 (22, 29), 5.2 (30), 5.3 (13)
Valine	5.92	5.01	4.41	6.5 (22), 5.4 (30), 6.6 (27)
Proline	4.75	4.00	3.60	5.6 (22), 5.1 (27), 5.5 (28)
Glutamic acid	16.50	14.49	9.78	16.9 (22), 16.6 (27), 16.95 (29)
Aspartic "	10.91	9.44	7.15	10.6 (22), 11.1 (27), 10.25 (29)
Alanine	6.25	4.99	6.12	
Threonine	5.83†	4.95	4.27	6.5 (22, 26), 6.2 (27), 6.3 (30)
Serine	4.23†	3.51	3.52	4.5 (22, 26), 4.9 (28)
Glycine	1.82	1.38	2.11	1.9 (22), 1.96 (29), 2.0 (28)
Arginine	5.90	5.29	11.80	6.2 (22, 26), 5.9 (27), 6.1 (30)
Lysine	12.82	11.25	15.30	12.4 (22), 12.4 (29), 12.3 (30)
Histidine	4.00	3.54	6.75	3.8 (22, 26), 3.35 (31), 4.1 (30)
Cystine + cysteine	6.52‡	5.54	4.73	
Tryptophan	0.58§	0.53	0.50	
Amide-NH_3	0.95‖		4.87	1.05 (22, 26)
Total		97.9	101.2	

* The value for methionine is that determined by Brand *et al.* (22, 26). The chromatograms gave a figure of about 0.92 (1).

† The average threonine and serine values of 5.55 and 3.81 from Table III have been divided by 0.95 and 0.90, respectively, in accordance with the estimates of Rees (17) for decomposition of these amino acids during hydrolysis.

‡ The cystine + cysteine value is that determined by Brand *et al.* (22, 26). The chromatograms gave an average value of 5.91.

§ The tryptophan value is that determined by Brand *et al.* (22, 26).

‖ This figure is a maximum value for amide-NH_3 calculated from the total NH_3 of the hydrolysate corrected for the approximate amount of NH_3 formed on the decomposition of serine and threonine.

Composition of Bovine Serum Albumin—The data on bovine serum albumin have been summarized in Table IV. The chromatograms gave a figure of 0.92 per cent for methionine (1) which is close to the value of 0.81

per cent as determined by Brand *et al.* (26). The same authors obtained a figure of 6.52 per cent for cysteine plus cystine which, in conjunction with the methionine content, accounted for 99.0 per cent of the sulfur of the protein. The average chromatographic value for cystine in the hydrolysate is 10 per cent below the above figure.

The total ammonia in the hydrolysate of bovine serum albumin (Table III) averages 1.06 per cent. Corrected for 0.068 per cent ammonia formed by the decomposition of serine and 0.040 per cent from threonine, the maximum possible figure for the amide ammonia of the protein becomes 0.95 per cent. This result, uncorrected for additional ammonia that might be formed as a result of the decomposition of other amino acids, has been used in Table IV. The amide ammonia determination of Brand (22) on bovine serum albumin gives the apparently high figure of 1.05 per cent.

The total nitrogen recovery in Table IV is 101.2 per cent. The weight recovery is 97.9 per cent. Possible causes for the discrepancy between the weight and nitrogen recoveries have already been mentioned.

Comparison of Chromatographic Results with Other Values—The results for the first six amino acids in Table IV have already been discussed (1). Additional values in Table IV include microbiological assays reported by Henderson and Snell (27), Gunness, Dwyer, and Stokes (13), Hier, Graham, Friedes, and Klein (30), and Velick and Ronzoni (28). As in the case of β-lactoglobulin, hydroxyproline has been shown to be absent (<0.006 per cent) in bovine serum albumin by Keston, Udenfriend, and Cannan.[1] For glutamic acid, arginine, and lysine, several values from the literature and the chromatographic results are all in agreement. The glycine figure, considering the small amount present, checks with the isotope dilution value of Shemin (29) and the microbiological assays (26, 28). The chromatographic value for aspartic acid is in agreement with the microbiological results (26, 27) and within 6 per cent of the isotope dilution value (29). The figure for proline is 5 to 15 per cent below the microbiological determinations (26–28). The values for threonine and serine are also lower than the earlier results. The chromatographic figure for histidine is in agreement with the results of the microbiological (30) and photometric (26) methods, but is higher than the value obtained by the isolation method of Vickery and Winternitz (31). Alanine has not previously been determined in bovine serum albumin.

SUMMARY

Chromatography of amino acids on starch columns has been applied to the determination of the composition of β-lactoglobulin and bovine serum albumin. A single chromatogram run with 1:2:1 n-butyl alcohol-n-propyl alcohol-0.1 N HCl distributes the amino acids to give an effluent curve which yields quantitative values for ten of the components of the hydro-

lysate. When the integrations of the overlapping peaks were included, a nitrogen distribution was obtained in each case which accounted for 100 ± 2 per cent of the total protein nitrogen. A combination of chromatograms run with three solvent systems is required for quantitative estimation of essentially all the components of the acid hydrolysates. About 2.5 mg. of protein are used per chromatogram. The complete series can be carried out in triplicate with a hydrolysate prepared from 25 to 50 mg. of protein.

To estimate the composition of the protein from data obtained on the hydrolysate, the values for serine and threonine must be corrected for the decomposition undergone by these amino acids during hydrolysis. Values for tryptophan and cysteine plus cystine determined by other methods are also required. The final tabulation of the results on β-lactoglobulin and bovine serum albumin has given total nitrogen recoveries of 99.6 and 101.2 per cent respectively, and weight recoveries of 97.7 and 97.9 per cent.

The individual amino acid analyses have been compared with values previously obtained by chemical, microbiological, and isotopic methods.

The authors wish to acknowledge the assistance of Miss Enid Mellquist and Mr. Anton Hornicek in the performance of this work.

BIBLIOGRAPHY

1. Stein, W. H., and Moore, S., *J. Biol. Chem.*, **176**, 337 (1948).
2. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **176**, 367 (1948).
3. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **178**, 53 (1949).
4. Palmer, A. H., *J. Biol. Chem.*, **104**, 359 (1934).
5. Sörensen, M., and Sörensen, S. P. L., *Compt.-rend. trav. Lab. Carlsberg, Série chim.*, **23**, 55 (1938).
6. Brand, E., Saidel, L. J., Goldwater, W. H., Kassell, B., and Ryan, F. J., *J. Am. Chem. Soc.*, **67**, 1524 (1945).
7. Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, **68**, 459 (1946).
8. Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C., *J. Biol. Chem.*, **160**, 35 (1945).
9. Tristram, G. R., *Biochem. J.*, **40**, 721 (1946).
10. Ryan, F. J., and Brand, E., *J. Biol. Chem.*, **154**, 161 (1944).
11. Haugaard, G., unpublished data cited by Stein, W. H., and Moore, S., *Ann. New York Acad. Sc.*, **47**, 95 (1946).
12. Smith, E., L., and Greene, R. D., *J. Biol. Chem.*, **172**, 111 (1948).
13. Gunness, M., Dwyer, I. M., and Stokes, J. L., *J. Biol. Chem.*, **163**, 159 (1946).
14. Keston, A. S., Udenfriend, S., and Cannan, R. K., *J. Am. Chem. Soc.*, **71**, 249 (1949).
15. Foster, G. L., *J. Biol. Chem.*, **159**, 431 (1945).
16. Chibnall, A. C., Rees, M. W., and Williams, E. F., *Biochem. J.*, **37**, 372 (1943).
17. Rees, M. W., *Biochem. J.*, **40**, 632 (1946).
18. McMahan, J. R., and Snell, E. E., *J. Biol. Chem.*, **152**, 83 (1944).
19. Cannan, R. K., Palmer, A. H., and Kibrick, A. C., *J. Biol. Chem.*, **142**, 803 (1942).
20. Warner, R. C., and Cannan, R. K., *J. Biol. Chem.*, **142**, 725 (1942).

21. Brand, E., and Kassell, B., *J. Gen. Physiol.*, **25**, 167 (1941).
22. Brand, E., *Ann. New York Acad. Sc.*, **47**, 187 (1946).
23. Gale, E. F., *Biochem. J.*, **39**, 46 (1945).
24. Li, C. H., *J. Am. Chem. Soc.*, **68**, 2746 (1946).
25. McMeekin, T. L., Polis, B. D., DellaMonica, E. S., and Custer, J. H., *J. Am. Chem. Soc.*, **70**, 881 (1948).
26. Brand, E., Kassell, B., and Saidel, L. J., *J. Clin. Invest.*, **23**, 437 (1944).
27. Henderson, L. M., and Snell, E. E., *J. Biol. Chem.*, **172**, 15 (1948).
28. Velick, S. F., and Ronzoni, E., *J. Biol. Chem.*, **173**, 627 (1948).
29. Shemin, D., *J. Biol. Chem.*, **159**, 439 (1945).
30. Hier, S. W., Graham, C. E., Friedes, R., and Klein, D., *J. Biol. Chem.*, **161**, 705 (1945).
31. Vickery, H. B., and Winternitz, J. K., *J. Biol. Chem.*, **156**, 211 (1944).

[Reprinted by permission of the American Chemical Society, copyright owner, from ANALYTICAL CHEMISTRY, April, 1949, Vol. **21**, No. 4, pp. 500–504]

APPARATUS FOR COUNTERCURRENT DISTRIBUTION

BY LYMAN C. CRAIG AND OTTO POST

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, June 17, 1948)

The possibility inherent in extraction as a tool for the separation and isolation of chemical compounds has been recognized almost from the beginning of chemistry. Although the procedure has been used as an analytical tool for investigating crude mixtures containing components with wide differences in solubility, further extension of its use to the analysis of mixtures with closely related components has been attempted only more recently.

The analogy of fractionation by distillation to fractionation by extraction has been considered (*9*) and it is only logical on the basis of the analogy to turn to some type of column extraction in order to gain the greatest efficiency and thus permit separations in spite of closely related partition ratios. On the other hand, attempts to develop small-scale continuous laboratory extraction columns for fractionation have met with great technical difficulties and the attempt has, in general, not been rewarding. For this reason the attention of the authors' laboratory, several years ago, was turned toward some type of discontinuous process with the hope that further information in regard to the requisites of quantitative extraction might be gained. Irrespective of this possibility, certain definite advantages of the discontinuous process, particularly for analytical purposes, have become apparent. These include an opportunity for the direct application of the type of calculation already developed in the mathematics of probability (*11, 14*). These mathematics have permitted a more precise interpretation of the results than is possible in the continuous case. The name "countercurrent distribution" has been given to the type of extraction that permits direct application of the binomial expansion in order to interpret the results.

Investigations along this line have now advanced to such a stage that a general method of considerable precision and wide applicability has been developed. The method has been particularly useful in proving purity and in characterizing substances that are difficult to study by the more established procedures. Types of substances studied have included synthetic antimalarials (*7*), the penicillins (*2, 6*), the streptomycins (*13*), fatty acids (*10*), purines and pyridines (*12*), and polypeptides of the gramicidin type (*8*).

For further development of the method it is the intention of this laboratory to consider mainly two separate lines of research. One involves the study of systems—i.e., the search for two immiscible phases (one may be a solid, *5*) which give the greatest possible differences in the partition ratios of the indi-

vidual members of a mixture. These may be spoken of as "selective" systems. Naturally, such systems must not interfere too greatly with the final isolation of the fractions. The other line of research is purely a technical one and involves the development of suitable apparatus for the performance of large numbers of quantitative extractions with speed and minimal labor. If a sufficient number of extractions could be applied, high selectivity of a system would not be required for many purposes.

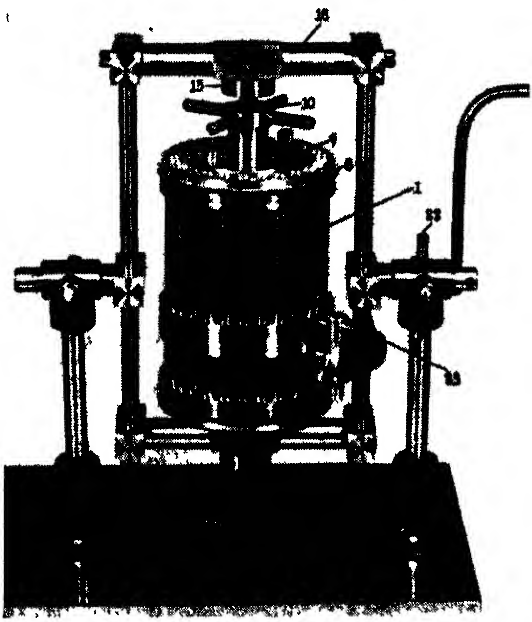


FIG. 1. 25-Tube Distribution Apparatus.

In order to achieve such multiple extractions, several types of apparatus offer possibilities. Up to the present time the best device from the analytical standpoint has proved to be a modification of the original countercurrent distribution machine (*4*). The present paper describes the model currently in use and mentions two other types of distribution apparatus which are under investigation.

Design of Apparatus

Figure 1 is a photograph of an apparatus containing 25 tubes. Figure 2 shows the same apparatus completely disassembled. The various parts will be described mostly from Figure 2.

All the parts of the apparatus which come in contact with the solutions are constructed from stainless steel and glass. The steel tubes of part 1, of 0.5-inch (1.25 cm.)

inside diameter and 4.75 inches in length, are joined to two circular steel plates as shown. The tubes pass through the steel plates with as perfect a fit as is mechanically possible, and are pressed in with considerable force. No detectable leakage has thus far been observed around the joints. The distance between adjacent tubes is approximately 0.125 inch; the over-all diameter of the part is approximately 6 inches. A hole slightly

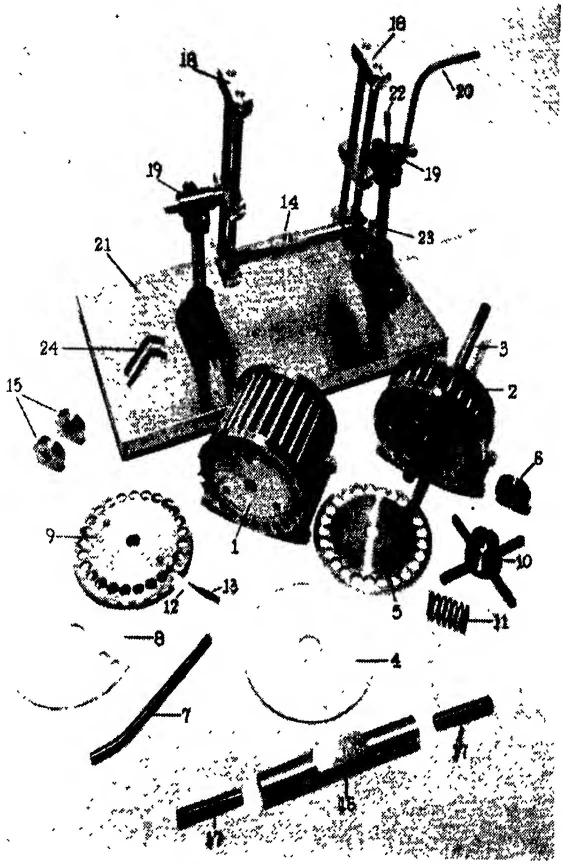


FIG. 2. 25-Tube Distribution Apparatus Disassembled.

larger than the central rod, 3, extends through the center of each end plate. Three aluminum studs join the two plates just inside the row of tubes and are held by three stainless steel Allen head cap screws whose heads can be seen on the plate of part 1. These make the part more rigid. The contacting surfaces of the two plates are ground flat with great accuracy. The tubes are numbered, 0 to 24, in counterclockwise direction on the top end plate.

Part 2 is constructed almost like 1, except that the tubes are only 2.5 inches in length and a heavy piece of aluminum is used for the reinforcement between the two

plates. The central rod, 3, is screwed into a heavy circular piece which in turn is held firmly on the plate by three Allen head cap screws. When 1 is placed over 2 as shown in Figure 1, its tubes can be made to coincide exactly with those of 2 by rotating the part around the central rod, 3. The tubes of 2 are numbered 0 to 24 in clockwise direction. The plate glass cover, 4, then seals each of the lower ends of the tubes of 2. The glass is held in position by the aluminum cover, 5, which in turn is held in position by the circular nut, 6. The steel rod, 7, is used for tightening the circular nuts.

The glass cover, 8, is then placed on the upper surface of 1 in order to close the remaining end of each of the tubes. It is held in position by the upper aluminum cover, 9, which in turn is held in position by the wing nut, 10. A spring, 11, fits beneath the wing nut, so that a slight pressure will be exerted on the cover even when the wing nut is loosened. The glass cover, 8, has two small holes on either side of the central hole and still another hole near its outer edge. The latter permits access to any tube of the apparatus during operation. Two short round rods extend from the under side of 9 and when 9 is placed over the glass cover, these rods extend barely through two corresponding holes of the glass cover. Thus when 9 is rotated around 3, the glass cover is also moved and in this way the opening may be brought over any tube desired. The hole of 9, which fits over the hole of 8, is cut out as shown and is sufficiently large so that a cover glass, 12, from a polariscope tube can be placed over the opening in 8. The cover glass is pressed firmly on the opening by a wedge, 13. The small end of the wedge is made from steel wire which fits into a tiny hole drilled into the inner edge of the cut-out hole of 9.

After the central part of the apparatus is assembled as described, it is placed so that the lower end of 3 extends through the hole in 14, where it is held firmly in position by one of the nuts, 15. The other nut is screwed lightly on the top of 3. The aluminum bar, 16, is placed in position between the two parts, 18, and the two steel plugs, 17, are inserted through the corresponding holes in 18 and into the two holes, one in each end of the bar. When the round nut, 15, is screwed upward, it presses against the bar and the unit is then ready for operation as shown in Figure 1. The assembled apparatus is supported by two bearings, 19, and can be tumbled by the crank, 20. The base of the apparatus, 21, is made of wood but is covered with stainless steel sheeting. The tubes of the apparatus can be held in an upright position by virtue of the plug, 22, which extends through one of the bearings.

When the wingnut, 10, is backed off a quarter turn, the upper sections of the tubes, 1, can be rotated over the lower sections at will, even though a certain pressure is exerted by the coil spring under the wing nut. The movable section can be stopped so that its tubes will exactly coincide with those of the stationary one by means of a machine plug, 23, which snaps into the shallow holes correctly placed in the lower end plate of 1. The machine plug is supported by screws in such a manner that it is adjustable with the small wrench, 24. The position of the tubes of the lower section can be adjusted by loosening nut 6.

In loading the apparatus for a distribution, parts 16, 15, 10, 9, and 8 are removed. The upper section is then rotated slightly until its tubes are midway from coinciding with the tubes of the lower section. Each tube of the upper section will now connect with two tubes of the lower section, and vice versa. All the tubes are thus connected at the sliding joint. Sufficient of the lower phase (previously equilibrated with the upper)

to fill all the lower sections, approximately 200 ml., is then poured into the top of one of the tubes of 1. After waiting a few minutes for the solution to flow into all the tubes, the upper section is rotated one complete circuit, until upper 0 is over lower 0. The arbitrary volume of the upper phase chosen for the run is then placed in each of the tubes. A hypodermic syringe is convenient for this purpose. If the sliding surface between 1 and 2 has been well seated there will be no leakage. The top parts, 8, 9, 10, 15, and 16, are now replaced and the instrument is ready for operation.

The sample to be distributed is placed in tube 0. After being brought into solution, equilibrium is reached by tumbling. A rather sudden inversion to a position approximately 150° from vertical, then back to 30° then to 150° and back again, etc., has been found to be the best method of tumbling for most cases. The tubes should be tipped in such a manner that the bubble of air moves from one end to the other of each tube with the greatest speed. It should reach the end of the tube at each stroke. The number of strokes required to reach complete equilibrium varies with the solute and the system (*1*), but usually is between 10 and 50.

At the end of the equilibration period, the phases are allowed to separate. Separation usually occurs more rapidly when the tubes are in the horizontal position, sometimes when they are at an angle of 45°, and less often when they are in the upright position. If the horizontal position is best, the tubes must be brought to the upright position slowly, in order not to cause dispersion again. As the end of each tube is of glass, the separation can be followed visually.

In order to make a transfer, the upright position is first ensured by the plug 22. The wing nut is backed off one quarter turn; the machine plug is withdrawn, and the upper section is rotated slightly in the clockwise direction. The plug is then allowed to snap back against the steel plate of 1. Upon rotating 1 further, 23 snaps into the next hole and the tubes are again superimposed. The wing nut is tightened and the apparatus is ready for a second equilibration. A system which equilibrates reasonably well will permit from 20 to 25 transfers per hour.

Aside from greater speed in operation as compared to the original design (*4*), the apparatus described here is more flexible. When the volume ratios desired of the two phases are not 1 to 1, a correspondingly longer or shorter section can be substituted for 1. Both longer and shorter sections have been successfully used. An apparatus with tubes of larger diameter can also be built.

An apparatus of the design of Figure 1, containing 54 tubes of 0.5-inch diameter, has been constructed and has been in successful operation for more than a year (Figure 3).

An interesting modification of this larger apparatus is that part 18 is itself mounted on a bearing at 25 and conveniently drops to the rear when the nut, 15, is released. Thus the top of the apparatus is completely clear of obstruction for filling, etc. In this apparatus, part of the central shaft is also removable by releasing a wing nut, 26, at the bottom. A hollow tube forms the lower part of the shaft from the top of part 2 to 26. The upper part of the shaft is held in position by a narrower extension which passes through the tube and further extends through 26. The spring, 11, of Figure 2 is not necessary for the larger apparatus.

With the 54-tube apparatus and 20 transfers per hour, some 2.7 hours are required for one complete circuit of the upper section and 1431 separate extractions are thus made. If a second circuit of the upper section is undertaken, the number of separate extractions made per hour remains constant at 20 × 54 = 1080. If the single withdrawal procedure is used, and the upper phase considered in discontinuous flow, this would give a flow rate, at 20 transfers per hour, of 340 ml. per hour when 17 ml. of the upper phase are used for each cell.

Among the many apparatus designs considered by the authors for multiple extractions, other than that of Figures 1 and 3, two deserve mention. One of

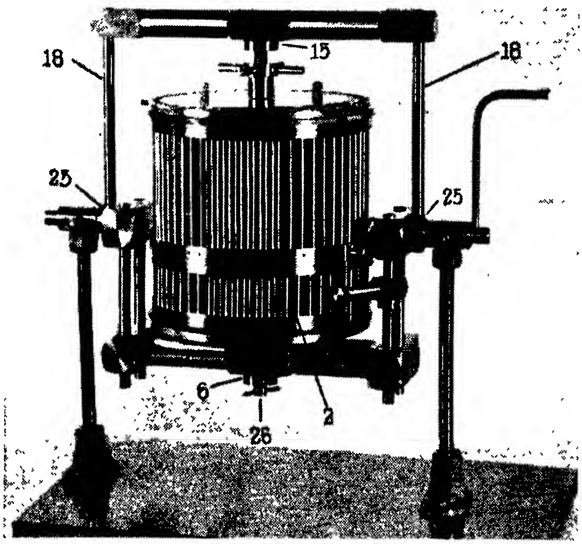


FIG. 3. 54-Tube Distribution Apparatus.

these, Figure 4, is particularly useful in studying mixtures preliminarily before taking the time for a more prolonged run in the apparatus described above.

The contacting units are ground glass stoppered tubes, 0.5 inch in inside diameter and 7.5 inches in length, and numbered 0, 1, 2 -- r. The tubes are held by spring clips attached to a Flexaframe rod. The stainless steel Gee clips, available from scientific supply houses, may be conveniently adapted. The rods attached to the clips are in turn attached to a longer rod which extends through two bearings on each end of the rod. The bearings are simply Flexaframe clamps in which the screw remains loose. The bearings are supported by two stands. A crank is attached to one end of the rod for tumbling the tubes.

In this design each tube receives its equal portion of stationary phase initially. The sample to be distributed is placed in tube 0. An arbitrary portion of the other phase, previously equilibrated with the first, is then introduced and equilibrium is reached by

inverting the tubes with the crank. Twenty-five inversions are usually sufficient (*1*). The glass stopper will stick sufficiently not to fall out, if it is given a slight turn when it is placed tightly in position. Small interchangeable stoppers are therefore more reliable than larger ones.

After the layers have separated, the phase to be moved is transferred to the adjoining tube, 1, by the small siphon shown in Figure 4, preferably made from stainless steel tubing. Pressing the rubber collar, through which the siphon extends, against the mouth of the tube permits actuation by either air pressure or vacuum. The glass tube to which the pressure or vacuum line is attached also passes through the rubber collar

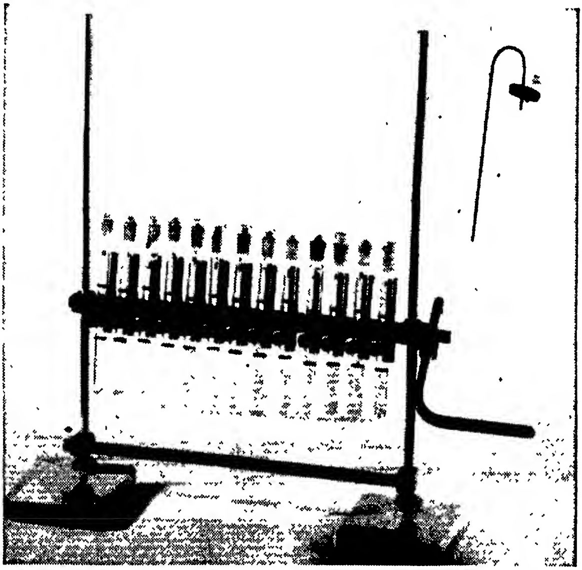


FIG. 4. Extraction Train Permitting Individual Transfers by a Siphon.

and has a hole at the bend. The operator's finger is placed over this hole, so that instant release of the vacuum or pressure is possible.

Following the first transfer, fresh mobile phase is added to 0 and an equilibration made. The mobile phase of 1 then goes to 2, that of 0 goes to 1, and fresh phase goes to 0. This procedure is continued until all the tubes of the series contain both phases—i.e., the fundamental operation is complete. A series of 12 tubes can be filled in this manner in approximately 1 hour.

The apparatus of Figure 4 is useful when stable emulsions tend to form, because the individual tubes can be placed directly in the centrifuge. Often only a single unit will emulsify and this can be withdrawn later from the series. Distributions which give initial difficulty, either from the tendency to emulsify or from volume shift due to a high concentration of solute, can be carried out in these tubes until the difficulty is overcome. The contents of each tube can then

be transferred to the corresponding tube of the machine of Figure 1 or 3 (after moving the upper section the corresponding number of stages).

An apparatus of the type of Figure 4 with larger units serves another useful purpose as a supplement to the apparatus of Figure 1 or 3. When considerable material to be fractionated is at hand, a 12- to 20-stage preliminary distribution is made with the individual tubes, for many times the volume of solvent here is possible. The solute in a selected tube is then recovered and subjected to

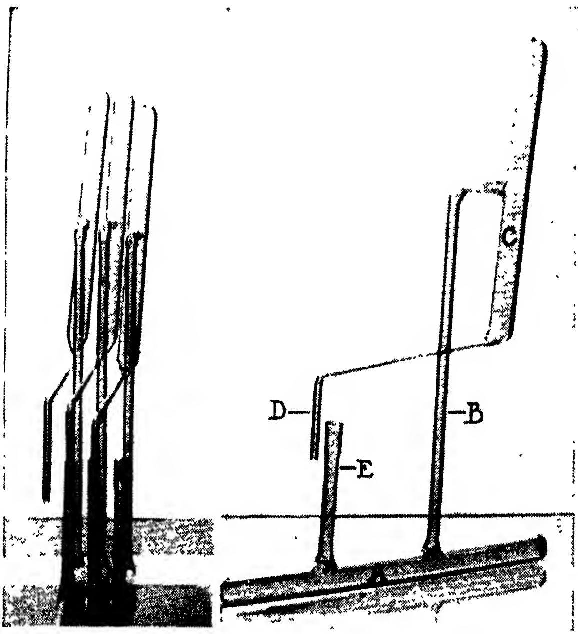


FIG. 5. Extraction Train Permitting Simultaneous Transfers by Decantation. Individual unit at right.

distribution in the apparatus of Figure 3, in which 200 to 300 transfers can be applied if desired. The combined separating power is thus considerable, although it is done at the expense of yield.

Figure 5 shows a third type of distribution apparatus under investigation in this laboratory. The single units interlock as shown at left, with the exit tube from one unit inserted into the entrance tube of its neighbor.

A is the equilibration chamber, which can be tilted at a 45° angle, then back to a 45° angle in the other direction without either of the phases splashing out through *E* or *B*. Tilting back and forth gives good dispersion of the two phases and equilibrium is reached exactly as with the other two types of countercurrent distribution apparatus described here.

After the phases have been allowed to separate, the unit is tilted somewhat more than 90° so that chamber *C* is lower than tube *B*. The upper phase will then decant through *B* into *C*, while the lower phase remains behind, as it is of such volume that it reaches *B* only when the apparatus is in the decanting position. When the unit is tilted back to the horizontal position, the upper phase in *C* will flow through *D* into the opening, E′, of the adjoining unit of the series. Fresh upper phase is then added through, *E*, etc. The units of a series can be individually supported by standard clamps and rods available in any laboratory.

Methods of Operation

In studies with the distribution apparatus thus far, four different methods of operation have proved most useful: (1) fundamental, (2) single withdrawal, (3) alternate withdrawal, and (4) completion of squares. Another method which would be of considerable interest from the theoretical standpoint might be some procedure which would introduce the principle of reflux. However, this must be left for a later occasion when a theoretical discussion is also presented.

Fundamental. In the fundamental procedure the apparatus is loaded as given in the previous section and when sufficient transfers have been applied so that upper O is over the tube adjacent to lower O on the right, the operation is complete. A fixed number of tubes, n thus permit $n-1$ transfers. The contents of each tube are withdrawn and analyzed for their solute content by some suitable analytical procedure. For purposes of simplification, the total content of a tube, both layers combined, is of primary interest, but often useful deductions may be derived by considering the layers separately. The amount of solute in each tube is plotted against the consecutive tube number to give the distribution pattern (*14*).

Single Withdrawal. If, when the state of affairs obtaining at the end of the fundamental procedure is reached, the process should be continued, a fast-moving band would be overtaking a slow-moving one and remixing of fractionated solute would occur. However, in certain cases where fast-moving bands are known to be absent, a second circuit of the upper section of the apparatus may be accomplished without remixing fractionated solute, because for practical purposes, all the solute will have been extracted from the advancing upper phase. In most cases a better procedure for the application of transfers of number greater than the total number of tubes consists of withdrawing the contents of a particular tube and substituting a fresh phase for the one withdrawn.

In the most simple of these modifications, the fundamental procedure is followed until upper 0 has reached the second tube from the right of lower 0, lower 52 in the apparatus of Figure 3. The contents of tube 53/53 are then withdrawn and discarded, since no solute can be present in these solvents as yet. The tube is left empty for the next transfer. When the upper section is advanced only the upper phase from tube

0/52 wil fall into the empty tube. However, at this point tube 53/0 will have no upper phase. The appropriate volume of fresh upper phase is accordingly added from a syringe through the opening in the cover and the equilibration is then performed. While the phases are separating, the upper phase present in tube 0/53 is withdrawn by means of a siphon. It constitutes the first member of the withdrawn series.

Another transfer again gives only upper phase advancing into the empty tube. It will be numbered 2 of the withdrawn series. The process may be continued until all the solute has been removed in the upper phase.

Often this process will simplify analytical difficulties, as only one phase need be dealt with. The use for a distribution, of a lower phase which otherwise would interfere with the final analysis, is thus permitted. The single withdrawal process is more nearly analogous to the collection of effluent fractions in chromatography than are any of the other procedures herein described. Rapid methods are available for calculating theoretical distributions for this type of operation. These will be treated in a forthcoming contribution.

If the upper phase should be the one that interferes with the analysis or if the solute of interest should favor the upper phase considerably, it may be desirable to withdraw from the trailing edge of the band. This may be accomplished just as easily as in the above case.

When the first transfer into the empty tube is made, fresh lower phase is added to tube 0/53 and an equilibration is made. While the phases are separating the lower phase present in tube 53/0 is withdrawn and numbered 1. On the next transfer, fresh lower phase is added to tube 0/0 and the lower phase present in tube 53/1 is withdrawn as number 2 of the series. This process may be continued until all the solute has been withdrawn.

In case the process is interrupted while solute yet remains in the apparatus, the distribution pattern would consist of two separate curves, as described in a previous paper of this series (*4*).

Alternate Withdrawal. In this procedure three separate patterns are obtained on a single run. One pattern represents a withdrawn series of the slower moving components—i.e., those of lower partition ratio—the second represents those of intermediate partition ratio, near 1 which remain in the machine, while the third represents the faster moving components of higher partition ratio. It, like the first, is a withdrawn series.

Specific directions for carrying out this procedure have been given (*6*). However, a modification is possible which gives only lower phase in the first withdrawn series and only upper phase for the third.

At first the fundamental procedure is followed and, as in the previous case, the first withdrawal step is made by allowing the upper phase from tube 0/52 to fall into an empty tube. Fresh lower phase is added then to tube 0/53 and an equilibration is made. While the phases are separating, the contents of 53/0 are set aside as the first tube

of the first withdrawn series. A transfer is then made and at this point fresh upper phase is added to tube 53/1. After equilibration the contents of tube 0/0 (upper phase only) are withdrawn and set aside as the first tube of the third series. On the next transfer, fresh lower phase is added to 1/0 and 0/1 is withdrawn as the second member of the first series. On the next transfer, fresh upper phase is added to 0/2 and 1/1 is withdrawn as the second member of the third series. This process can be continued as long as desired. At the conclusion of the run the tubes in the apparatus are represented by the second curve in the series.

Completion of Squares. The method of carrying out this procedure has been outlined by Bush and Densen (*3*).

Care and Service of the Apparatus

It is obvious that the sliding surfaces of sections 1 and 2 of Figure 2 must be kept free from grit, pieces of broken glass, or steel fragments. In particular, when the machine is first received from the maker a thorough cleaning is advisable. Machine cuttings cling tenaciously and are very difficult to remove entirely.

Each section should be placed on a wooden support in the sink and each tube washed separately with a tube brush which is slightly larger than the diameter of the tube of the machine. While each tube is being scrubbed with the brush, a good stream of lukewarm water should be maintained through the tube. The stream of water can come directly from the tap through a rubber tube which is pressed firmly on the tube opening opposite that from which the brush is inserted. The wire part of the brush should not come in contact with the polished surfaces.

Finally, the section should be rinsed with alcohol or acetone and permitted to dry. It has been found a good procedure for the operator to rub or wipe all the polished surfaces with his finger tips. Clean hands are required for this operation. Apparently tiny bits of steel are picked up by the skin of the fingers during this operation, though the particles are not of size large enough to penetrate the skin. A clean towel can be used for wiping the hands from time to time during the process. Wiping the surfaces with a clean finger is always advisable just prior to placing the two surfaces together.

Part 1 always should be placed on part 2 with great care in order to avoid shock. It then should be moved gently in either direction and should slide smoothly. If not, a particle of grit may be suspected and the surfaces must be gone over again with a clean finger. Little difficulty has thus far been experienced in this laboratory in keeping the surfaces sufficiently clean. A surface, once in satisfactory operation, requires regrinding only after many runs.

If a particle of grit or steel does enter during the run, it will immediately cause the machine to leak. A circular score will be noted on both surfaces when the machine is taken apart. This is not a serious misfortune unless the score is very deep. The difficulty often may be remedied as follows:

A hole is cut in the center of a sheet of fine crocus cloth slightly larger than the

diameter of the apparatus. This is placed between the sliding surfaces and the upper section gently revolved a few times. The cloth is then reversed, so that the abrasive is in contact with the other section. If this treatment does not stop the leakage, regrinding is advisable. Regrinding with fine emery requires approximately 2 hours.

A steel rod 0.25 inch in diameter and 18 inches in length is the only apparatus required. The grinding compound is made by suspending the finest Turkish emery in Carbitol. The central rod, 3 of Figure 2, is removed together with the small circular plates which support 2. Section 1 is placed on a clean towel with the side to be ground facing upward. A limited amount of the grinding suspension is then spread rather evenly over the surface with a soft brush. Section 2 is placed gently on 1 with the surface to be ground facing downward, and it is rotated gently to and fro in order to ensure a fairly even distribution of the emery. The steel rod is thrust through the central holes of section 2 but only through the upper hole of section 1. The upper end of the steel rod is then grasped loosely and pushed in the plane of the surfaces as far as it will go without moving the lower section. The upper section, however, has been moved so that half or slightly more of the openings of tubes of the lower section have been uncovered on one side. By moving the upper end of the rod in a circular motion in the plane of the surfaces, a circular grinding action will be produced. The widest circle permitted by the rod and the hole in the lower section should be employed. When the rod is held only loosely, it will revolve in the hand and, furthermore, the upper section not only will move in a circular motion back and forth across the lower part but will also steadily progress from one tube to the next around the circle of tubes of the lower section. This is the effect desired. The motion should be smooth, never jerky.

Fresh emery is added from time to time. At first the film of emery is wiped clean from certain spots which become uncovered at each stroke of the grinding action. These are the high spots. Later the upper section will appear to slide more evenly on the lower and a uniform film of the emery will cover all the surface. The grinding operation is nearly finished at this point but the grinding is continued somewhat longer. Upon washing, the deeper circular scores in the polished surfaces will still be visible, but these will not usually interfere. If leakage still persists a preliminary grind in which Carborundum is employed, may be required.

When the number of individual units employed in multiple extraction becomes high, the mere washing of the tubes becomes a significant problem. From this standpoint the design of Figure 1 or 3 is extremely good, for here all the tubes are washed as a unit after a routine run.

In washing the larger apparatus of Figure 3, a large shallow pan which is not too wide to pass between the supports is placed under the apparatus. After the glass cover is removed, the upper section is turned midway from the position of coincidence of the tubes and sufficient distilled water or solvent to more than fill the lower section is poured in. When the solvent has flowed equally into all the tubes the upper section is lifted slightly. This permits the solvent to gush out through the sliding joint but will leave the lower section full. The upper section is again seated and the cover replaced. The tubes are tumbled a few times, then the glass cover is removed. The apparatus is

carefully tilted so that all the solvent will flow from the tubes and drop into the pan. Proper care should be taken during this operation that the upper section does not slide off the central stem and that it is seated against the lower section before the whole is righted again.

Several rinsings made as above may be required. However, the final rinse should be with acetone or alcohol. Ordinarily the lower glass plate is not taken off after a distribution, but from time to time this is required for a more thorough cleaning.

LITERATURE CITED

(1) Barry, G. T., Sato, Y., and Craig, L. C., *J. Biol. Chem.*, **174,** 209 (1948).
(2) *Ibid.*, **174,** 221 (1948).
(3) Bush, M. T., and Densen, P. M., ANAL. CHEM., **20,** 121 (1948).
(4) Craig, L. C., *J. Biol. Chem.*, **155,** 519 (1944).
(5) Craig, L. C., Golumbic, C., Mighton, H., and Titus, E., *Science,* **103,** 587 (1946).
(6) Craig, L. C., Hogeboom, G. H., Carpenter, F. H., and du Vigneaud, V., *J. Biol. Chem.*, **168,** 665 (1947).
(7) Craig, L. C., Mighton, H., Titus, E., and Golumbic, C., ANAL. CHEM., **20,** 134 (1948).
(8) Gregory, J. D., and Craig, L. C., *J. Biol. Chem.*, **172,** 839 (1948).
(9) Saal, R. N. J., and Van Dyck, W. J. D., First World Petroleum Congress, London, *Proc.*, **2,** 352 (1933).
(10) Sato, Y., Barry, G. T., and Craig, L. C., *J. Biol. Chem.*, **170,** 501 (1947).
(11) Stene, S., *Arkiv. Kemi. Mineral. Geol.*, **18A,** No. 18 (1944).
(12) Tinker, J. F., and Brown, G. B., *J. Biol. Chem.*, **173,** 585 (1948).
(13) Titus, E. A., and Fried, J., *Ibid.*, **168,** 393 (1947), **174,** 57 (1948).
(14) Williamson, B., and Craig, L. C., *Ibid.*, **168,** 687 (1947).

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, June, 1949, Vol. 179, No. 2, pp. 623–632]

THE VERATRINE ALKALOIDS

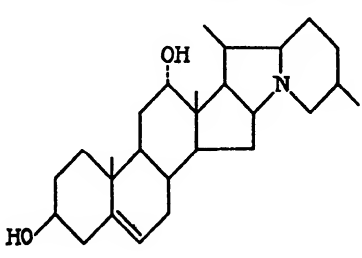
XXIX. THE STRUCTURE OF RUBIJERVINE

BY YOSHIO SATO AND WALTER A. JACOBS

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, February 15, 1949)

Rubijervine has been shown to be a tertiary steroid base of 3(β)-hydroxy-Δ^5-stenol character. This was supported by its formulation, $C_{27}H_{43}O_2N$, by the formation of a digitonide, by its hydrogenation to a dihydro derivative, and by its oxidation to a Δ^4-ketone, which in turn could be reduced to epimeric 3(α)- and 3(β)-Δ^4-stenols which gave characteristic Rosenheim reactions (1–3). The presence of a second hydroxyl group was shown by the formation of a basic diacetyl derivative (2). On dehydrogenation with selenium, rubijervine yielded the characteristic 2-ethyl-5-methylpyridine obtained from other veratrine alkaloids (4–6) and from solanidine (2, 7). Contrary to the latter, however, as in the case of the other veratrine bases, no Diels' hydrocarbon was isolated. Instead, an isomeric methylcyclopentenophenanthrene (1), not previously encountered, was obtained which has not been identified with certainty but appeared to agree in properties with synthetic α-methyl-1,2-cyclopentenophenanthrene (8). In addition an appreciable amount of a phenolic hydrocarbon was obtained for which the formulation, $C_{18}H_{16}O$, was derived and which appeared to be a derivative of a methylcyclopentenophenanthrene (1). This dehydrogenation product was not isolated in the case of any of the other bases and thus far is presumably a product characteristic of rubijervine and probably due to its extra hydroxyl group. More recently in work undertaken to remove this hydroxyl it has been definitely shown, by its conversion into solanidine and into solanidanol-(3β) (9), that rubijervine is a hydroxysolanidine. From the general evidence, the most satisfactory interpretation for the structure of rubijervine, barring possible epimerizations, appears to be a 12(α)-hydroxysolanidine (Δ^5-solanidene-3(β), 12(α)-diol), Formula I.



I

A number of preliminary attempts to prepare rubijervine monoacetate resulted in the preponderant formation of the diacetate, and this was abandoned in favor of benzoylation when the latter proved more satisfactory. On benzoylation rubijervine yielded varying proportions, depending upon the conditions used, of *rubijervine-3-benzoate* and *rubijervine dibenzoate* which could be separated chromatographically. That the 3-hydroxyl group was more readily benzoylated with the initial preferential formation of the 3-benzoyl derivative was shown by the failure of the monobenzoate to form a digitonide, and by the fact that its protection led to the production of solanidine. Oxidation of the monobenzoate with chromic acid yielded the *monoketobenzoate* (*Δ^5-solanidene-3(β)-ol-12-one benzoate*), which could be readily saponified to *rubijervone-12* (*Δ^5-solanidene-3(β)-ol-12-one*). The benzoate, however, was used for conversion into the *semicarbazone*, which in turn was reduced by the Wolff-Kishner method. The reaction product, after purification through alumina, yielded a base which agreed in all properties such as melting point and rotation with solanidine. This was confirmed by the comparison of its acetyl derivative with acetylsolanidine.

In the conversion of rubijervine into solanidanol-(3β), dihydrorubijervine (2) was oxidized with chromic acid to the *diketo derivative* (*solanidane-3,12-dione*). The *disemicarbazone* of the latter, when subjected to the Wolff-Kishner reduction, yielded a mixture from which solanidanol-(3β) was isolated in fair yield. Along with the latter a small amount of a by-product was obtained which was believed to be solanidane. The anomalous behavior of the 3-semicarbazone group, in that it was reduced to the hydroxyl group, is in agreement with the observations made by Dutcher and Wintersteiner (10). These experiments, barring rearrangement, have shown that rubijervine must possess the same configuration as solanidine but with an extra secondary hydroxyl group.

In the course of the work *dihydrorubijervine dibenzoate* and *dihydrorubijervine-3-benzoate* were also prepared. The latter was oxidized to *solanidane-3(β)-ol-12-one benzoate*, and unsuccessful preliminary attempts were made to dehydrogenate this substance partially to a possible $\Delta^{9(11)}$-12-keto derivative with selenious acid in accordance with the procedure of Schwenk and Stahl (11).

In an effort to locate the position of the extra secondary hydroxyl group, a series of studies was made. The ready formation of a monoacyl derivative and the lack of ring cleavage on mild oxidation seemed to exclude positions 2 and 4, vicinal to the 3(OH) group. Oxidation with periodic acid was nevertheless attempted on rubijervine but, as expected, no reaction was observed. The stability of the diketone observed with alkali, its absorption spectrum (Fig. 1), and the non-formation of a pyri-

dazine derivative (12) with hydrazine also preclude the presence of the OH group on position 1.

The stability of rubijervine towards acids and the apparent lack of an α,β-unsaturated carbonyl group in the 3-hydroxyketo compound (Δ^5-solanidene-3(β)-ol-12-one), as shown by the absorption spectrum (Fig. 1), makes assignment of OH to position 7 likewise untenable. In view of the accepted inactivity of the carbonyl group at position 11 towards ketonic reagents (13), this position can also be eliminated, since the diketodihydro derivative (solanidane-3,12-dione) forms the disemicarbazone. This leaves as possibilities only position 12 in Ring C, position 15 in Ring D, and

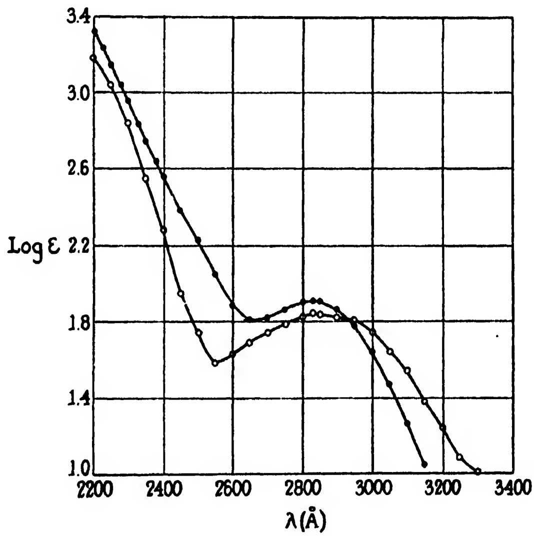


FIG. 1. ●, Δ^5-solanidene-3(β)-ol-12-one; ○, solanidane-3,12-dione; both in ethanol

position 23 or 24 in the heterocyclic portion (position 26 is eliminated because such a carbonyl derivative would be a substituted neutral lactam).

Whereas solanidine yields Diels' hydrocarbon upon selenium dehydrogenation, an isomer was obtained from rubijervine. If this result is due in some way to the interference offered by the second OH group towards the normal shift of the angular methyl group, hydroxyls located on Ring C or D might seem more likely to interfere with such a shift than those on the N ring. In such a case, positions 12 and 15 would appear to be the most likely for the second hydroxyl group. Some evidence seems to favor the 12 position. Considerable difficulty was encountered in the preparation of the semicarbazone of the ketomonobenzoate and disemicarbazone of the diketodihydro derivative. Analyses of the samples invariably showed a low nitrogen content and only by a purification which

involved considerable loss of material could satisfactory analytical figures be obtained. This is somewhat in accord with the findings of Dutcher and Wintersteiner (10) that ketones with carbonyl at position 12 can, on treatment with semicarbazide acetate, lead to incomplete semicarbazone formation.

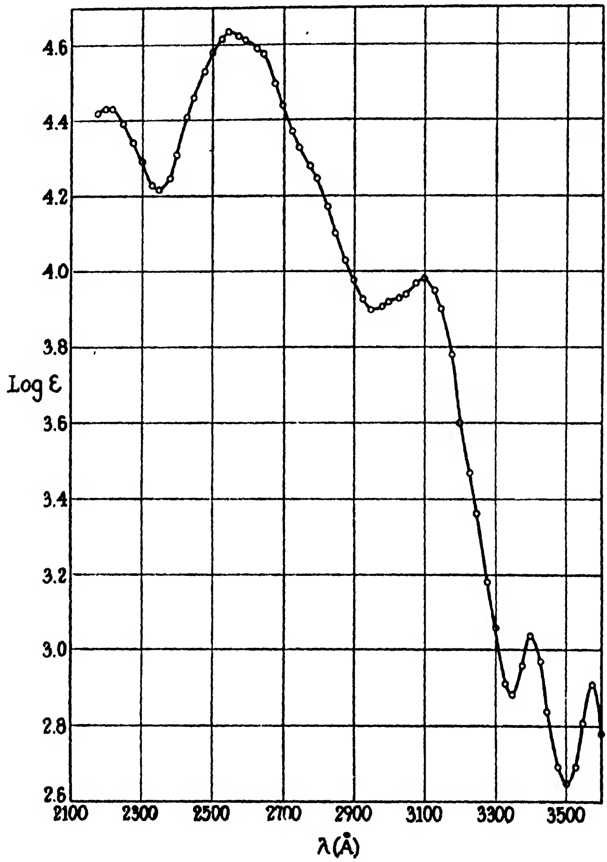


Fig. 2. Absorption curve of $C_{18}H_{16}O$ from rubijervine; in ethanol

Optical rotation data also seem to favor the 12 position on applying Barton's (14) method of molecular rotation differences. The molecular rotation of dihydrorubijervine, $[M]_D = +220°$, less the rotation of solanidanol-3β) (9), $[M]_D = +113°$, is +107°. This is in fairly good agreement with the value listed by Barton for a 12(α)OH, *viz.*, +93°. Also the rotation for the diketodihydro derivative, $[M] = +489°$, less the rotation for solanidane-3-one, $[M]_D = +182°$, gives +307°, which is compatible with the data given by Barton for the 12-keto group of +270° (14).

Finally, in the phenolic dehydrogenation product of rubijervine $C_{18}H_{16}O$, (ultraviolet absorption shown in Fig. 2) apparently characteristic of the latter, the favored interpretation is that the phenolic group comes from the extra hydroxyl of the base. If so, this would exclude any position on Ring D or the side chain but not position 12. Although the data presented favor such a 12(α)-hydroxyl group, further studies are necessary to make this conclusion final.

EXPERIMENTAL

Rubijervine Monobenzoate and Dibenzoate—In a typical experiment 500 mg. of rubijervine were dissolved in 5 ml. of dry benzene and 5 ml. of dry pyridine by slight warming and 0.3 ml. of benzoyl chloride was added to the solution. This was then heated in an oil bath for 50 minutes at 100° (±5°). Most of the solvent was removed under diminished pressure to a syrupy consistency and 50 ml. of water were added. Addition of a slight excess of dilute ammonia resulted in a voluminous precipitate which was extracted with chloroform. The extract was washed with water and dried. After the chloroform was removed, the crude residue was dissolved in chloroform-benzene (1:10) and chromatographed through 25 gm. of (Brockmann's) alumina. Elution with the same solvent mixture (1:10) yielded at first the dibenzoate. When this was changed to a 1:2 solvent mixture, the monobenzoate followed.

The monobenzoate crystallized as needles from benzene which melted under the microscope at 260–262°. Yields generally averaged about 80 per cent.

$C_{34}H_{47}O_3N$. Calculated, C 78.87, H 9.15; found, C 78.77, H 9.15

The dibenzoate also crystallized as needles from methanol-acetone with a micro melting point of 186–187.5°.

$C_{41}H_{51}O_4N$. Calculated, C 79.19, H 8.27; found, C 79.31, H 8.56

By raising the temperature or increasing the time of heating, the yield of the dibenzoate was increased considerably.

Δ^5-Solanidene-3(β)-ol-12-one Benzoate—A solution of 37 mg. of CrO_3 in 4 ml. of 90 per cent acetic acid was added dropwise to 260 mg. of rubijervine monobenzoate dissolved in 3 ml. of the same solvent and maintained at about 15°. An immediate precipitate formed which redissolved on agitation. After standing 16 hours at room temperature, about two-thirds of the solvent was removed under diminished pressure. 75 ml. of H_2O were then added to the viscous syrup, followed by neutralization with dilute ammonia. The copious precipitate was extracted with chloroform and the extract was washed with water and dried over anhydrous Na_2SO_4. After removal of the solvent, the residue was crystallized from acetone.

It formed long rods which melted at 214–216° and solidified into large slabs or blades which again melted at 233–236°. For analysis it was dried at 110° and 0.2 mm.

$C_{34}H_{45}O_3N$. Calculated, C 79.18, H 8.80; found, C 79.16, H 8.82

In several runs yields of 70 to 75 per cent were obtained.

Δ^5-Solanidene-3(β)-ol-12-one—42 mg. of the above benzoate were dissolved in 0.8 ml. of benzene and refluxed gently with 10 ml. of 1 per cent methanolic KOH solution for 25 minutes. When about two-thirds of the solvent were removed under reduced pressure, needles separated. When twice recrystallized from methanol-ether, it formed long silky needles which melted under the microscope at 236–238°.

$$[\alpha]_D^{28} = +45° \ (c = 0.89 \text{ in chloroform})$$

$C_{27}H_{41}O_2N$. Calculated, C 78.77, H 10.05; found, C 78.60, H 10.09

The ultraviolet absorption spectrum of the substance shows only carbonyl absorption, as given in Fig. 1.

Semicarbazone of Δ^5-Solanidene-3(β)-ol-12-one Benzoate—180 mg. of the keto benzoate in benzene were concentrated to dryness *in vacuo* to insure removal of any acetone of crystallization and then dissolved in 50 ml. of warm 95 per cent ethanol. A semicarbazide acetate solution was prepared from 0.2 gm. of potassium acetate in 2 ml. of 95 per cent ethanol and 0.2 gm. of semicarbazide hydrochloride in 0.6 ml. of H_2O. The filtrate from precipitated KCl was added to the above solution. The mixture was refluxed for 3 hours and allowed to stand overnight. After removal of about two-thirds of the solvent under reduced pressure, 50 ml. of H_2O were added and the solution was made slightly alkaline with ammonium hydroxide. The resulting copious precipitate was extracted with chloroform and the solution was thoroughly washed with water and dried over Na_2SO_4. The white solid obtained from the extract could not be crystallized. The amorphous residue (220 mg.) was thoroughly digested with cold ether, collected, and dried at 110°. 132 mg. of white amorphous solid were recovered, which decomposed above 265° with preliminary discoloration. In four runs the analyses all indicated low C and N values, even when pyridine was used as the solvent, as recommended by Dutcher and Wintersteiner (10). A satisfactory analysis was obtained only after the material was thoroughly washed with ether with a considerable loss of the substance.

$C_{35}H_{48}O_3N_4$. Calculated, C 73.39, H 8.45, N 9.78
Found, " 73.30, " 8.42, " 9.93

Reduction of Semicarbazone to Solanidine—125 mg. of the above semicarbazone placed in a bomb tube with sodium ethylate solution pre-

pared from 125 mg. of sodium and 5 ml. of absolute alcohol were heated for 8 hours at 200–210°. The contents of the tube were mixed with 100 ml. of H_2O and the opaque solution was acidified with dilute HCl. A practically clear solution resulted which was extracted thoroughly with ether to remove benzoic acid. 20 mg. of the latter were recovered. The aqueous phase was next made alkaline with ammonium hydroxide and the resulting precipitate was extracted with ether. The washed and dried extract, after removal of ether, yielded 60 mg. of white solid, which was dissolved in benzene and chromatographed through 3 gm. of alumina. 20 mg. of substance were eluted with benzene-ether (9:1). When this was recrystallized twice from acetone, needle-shaped crystals were obtained which melted under the microscope at 214–218°. This substance gave no depression in melting point with an authentic specimen of solanidine (micro melting point 215–218°).

$$[\alpha]_D^{28} = -27° \ (c = 0.36 \text{ in chloroform})$$

Prelog and Szpilfogel reported $[\alpha]_D = -27.0° \pm 4°$ (9).

$C_{27}H_{43}ON$. Calculated, C 81.54, H 10.91; found, C 81.51, H 10.85

In another run the crude substance obtained from the Wolff-Kishner reduction was directly crystallized from acetone and was used for the following acetylation.

32 mg. of the base dissolved in 0.8 ml. of pyridine were treated with 0.4 ml. of acetic anhydride and allowed to stand for 17 hours at room temperature. The bulk of the solvent was removed *in vacuo* and 25 ml. of H_2O were added to the residue. The aqueous phase was made slightly alkaline with dilute ammonia and the flocculent mass was extracted with ether. The latter left a residue which, when recrystallized twice from alcohol, formed flat blades which melted at 207–209°.[1] This showed no depression in a mixed melting point with authentic acetylsolanidine.

$C_{29}H_{45}O_2N$. Calculated, C 79.04, H 10.30; found, C 79.20, H 10.32

Dihydrorubijervine Monobenzoate and Dibenzoate—0.2 ml. of benzoyl chloride was added to 0.175 gm. of dihydrorubijervine (2) dissolved in 5 ml. of benzene and 5 ml. of pyridine. The solution was heated in an oil bath at 110–120° for 75 minutes. After partial concentration and addition of water, the solution was made alkaline with dilute ammonia and the precipitated mass was filtered and dried. This material was chromatographed over alumina. The dibenzoate (120 mg.) was obtained by elution with benzene-ligroin (3:1), while the monobenzoate (35 mg.) followed in the benzene-ether (2:1) eluate.

[1] Schöpf and Herrmann report 206–208° (15).

The dibenzoate crystallized from benzene as needles which melted at 266–269°.

$C_{41}H_{53}O_4N$. Calculated, C 78.93, H 8.56; found, C 79.06, H 8.47

The monobenzoate was crystallized from methanol-acetone. It formed rosettes of needles and melted at 187–190°.

$C_{34}H_{49}O_3N$. Calculated, C 78.57, H 9.50; found, C 78.70, H 9.46

Solanidane-3(β)-ol-12-one Benzoate—30.5 mg. of chromic oxide dissolved in 3.5 ml. of 80 per cent acetic acid were added dropwise to 156 mg. of the above monobenzoate in 2 ml. of acetic acid. The mixture was allowed to stand at room temperature for 80 minutes. After concentration, 125 ml. of H_2O were added, followed by excess dilute ammonia. The voluminous mass was extracted with chloroform. The washed and dried extract yielded, after removal of the solvent, the crude product which was crystallized from a mixture of acetone and benzene. It formed rosettes of needles which melted at 236–241°.

$C_{34}H_{47}O_3N$. Calculated, C 78.87, H 9.15; found, C 78.77, H 9.18

The above compound was treated with selenious acid according to the procedure of Schwenk and Stahl (11), but nothing definite could be isolated from the darkly colored solution.

Solanidane-3,12-dione—0.25 gm. of CrO_3 dissolved in 10 ml. of 80 per cent acetic acid was added dropwise to 0.31 gm. of dihydrorubijervine (2) dissolved in 25 ml. of glacial acetic acid. After 1 hour at 27–29°, the mixture was diluted and extracted with chloroform. The chloroform phase was washed with 5 per cent $NaHCO_3$, then with H_2O, and dried over Na_2SO_4. After removal of the solvent, the crude product was crystallized from acetone. It formed large rhombic crystals which probably contained solvent. When viewed under the microscope, it melted at about 215° and then solidified as small platelets which melted again at 242–244°. It was dried at 110° and 0.2 mm. pressure. The ultraviolet absorption spectrum of the compound is shown in Fig. 1.

$[\alpha]_D^{25} = +119°$ (c = 1.19 in chloroform)

$C_{27}H_{41}O_2N$. Calculated, C 78.78, H 10.05; found, C 78.70, H 9.95

When this substance was refluxed in methanolic alkali for 2 hours, it was recovered unchanged.

Disemicarbazone—The procedure of Dutcher and Wintersteiner was closely followed (10). A solution of 500 mg. of the above diketodihydro derivative in benzene was evaporated to dryness *in vacuo* to remove any solvent of crystallization. 500 mg. of semicarbazide hydrochloride dissolved in 1.5 ml. of H_2O were treated with a solution of 500 mg. of potassium acetate in 5 ml. of absolute alcohol. The filtrate from KCl was

added to the above diketodihydro derivative dissolved in 10 ml. of pyridine and 8 ml. of absolute alcohol. After the addition of 5 ml. of water, the mixture was gently warmed on the steam bath for a half hour and 3 ml. of chloroform were then added to dissolve the precipitated solid. After standing for 70 hours, the mixture was poured into 200 ml. of H_2O and extracted with chloroform. When the crude chloroform residue was thoroughly washed with ether and dried, 540 mg. of a slightly colored amorphous substance were obtained. It began to discolor at about 260° and charred completely at 300°. When this was directly analyzed without further purification, the nitrogen value was low, a result obtained in four runs. Satisfactory figures resulted only when the material was dissolved in hot alcohol-water and allowed to separate slowly by gradual cooling. About half of the material was lost in this purification.

$C_{29}H_{47}O_2N_7$.	Calculated.	C 66.25,	H 9.01,	N 18.65
	Found.	" 66.10,	" 9.16,	" 18.30

Wolff-Kishner Reduction of Disemicarbazone—A mixture of 0.25 gm. of sodium dissolved in 7 ml. of absolute ethanol and 280 mg. of the disemicarbazone was heated in a bomb tube for 8 hours at 200° ± 10°. The contents of the tube were poured into 100 ml. of water and extracted with ether. Difficulty was encountered because of emulsification. Subsequent extraction with chloroform proved less troublesome. After removal of the solvents, the material in each case was crystallized from acetone. From the mother liquor of each fraction a small amount of substance which melted at 272–274° was obtained, but it was not investigated further. Since the main fractions were not homogeneous, they were recombined (65 mg.) and chromatographed through 3 gm. of alumina. Elution with benzene yielded in succession two components, a low melting fraction (28 mg.) and a higher melting one (37 mg.). The latter, when recrystallized twice from acetone, formed long needles, which melted at 218–220° and showed no depression when mixed with authentic solanidanol-(3β).

$[\alpha]_D^{30} = +31°$ (c = 0.70 in chloroform); $[\alpha]_D = +28.2°$ (±4°) was reported by Prelog and Szpilfogel (9) for solanidanol-(3β).

$C_{27}H_{45}ON$. Calculated, C 81.14, H 11.35; found, C 81.20, H 11.20

The above low melting fraction crystallized as rosettes of needles from 95 per cent ethanol, which melted at 164–166°.[2] This substance is believed to be solanidane. Unfortunately no analysis was obtained with it.

$$[\alpha]_D^{23} = +36° \ (c = 0.43 \text{ in chloroform})$$

$[\alpha]_D^{17} = +33.1°$ (±2°) was reported by Prelog and Szpilfogel (9).

[2] Bergel and Wagner give 163–164° (16); Dieterle and Rochelmeyer give 164–165° (17).

All analytical work was performed by Mr. D. Rigakos of this laboratory.

SUMMARY

Rubijervine has been shown to be solanidine with an additional OH group by the conversion of the monoketo derivative of rubijervine into solanidine and by the conversion of the diketodihydro derivative of rubijervine into solanidanol-(3β). Possible positions for the second hydroxyl group have been discussed and the provisional conclusion reached that it is a 12(α)-hydroxyl group.

BIBLIOGRAPHY

1. Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **148**, 41 (1943).
2. Craig, L. C., and Jacobs, W. A., *J. Biol. Chem.*, **149**, 451 (1943).
3. Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **159**, 617 (1945).
4. Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **124**, 659 (1938).
5. Jacobs, W. A., Craig, L. C., and Lavin, G. I., *J. Biol. Chem.*, **141**, 51 (1941).
6. Craig, L. C., and Jacobs, W. A., *J. Biol. Chem.*, **143**, 427 (1942).
7. Prelog, V., and Szpilfogel, S., *Helv. chim. acta*, **25**, 1306 (1942).
8. Ruzicka, L., Ehmann, L., Goldberg, M. W., and Hösli, H., *Helv. chim. acta*, **16**, 833 (1933).
9. Prelog, V., and Szpilfogel, S., *Helv. chim. acta*, **27**, 390 (1944).
10. Dutcher, J. D., and Wintersteiner, O., *J. Am. Chem. Soc.*, **61**, 1992 (1939).
11. Schwenk, E., and Stahl, E., *Arch. Biochem.*, **14**, 125 (1947).
12. Windaus, A., *Ber. chem. Ges.*, **39**, 2249 (1906).
13. Steiger, A., and Reichstein, T., *Helv. chim. acta*, **20**, 817 (1937).
14. Barton, D. H. R., and Klyne, W., *Chem. and Ind.*, **48**, 755 (1948).
15. Schöpf, C., and Herrmann, H., *Ber. chem. Ges.*, **66**, 298 (1933).
16. Bergel, F., and Wagner, R., *Ber. chem. Ges.*, **66**, 1096 (1933).
17. Dieterle, H., and Rochelmeyer, H., *Arch. Pharm.*, **273**, 539 (1935).

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, August 1949, Vol. 180, No. 1, pp. 133–144]

THE ACONITE ALKALOIDS

XXII. THE DEMETHYLATION OF DELPHININE DERIVATIVES

BY WALTER A. JACOBS AND YOSHIO SATO

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, March 31, 1949)

Characteristic of the poisonous group of aconite alkaloids such as delphinine and aconitine is the presence in the molecule of four methoxyl groups. It was obvious from the start that demethylation would be necessary to facilitate their structural study in certain directions. In the earliest studies of this laboratory, repeated attempts were made to demethylate the bases themselves or certain of their derivatives with hydriodic acid and phosphorus or aluminum chloride, but the results proved unpromising in the cases studied. On the other hand, instances of partial demethylation (1) were encountered, mostly in the use of hydrochloric acid and in a few cases with nitric acid. Thus with α-oxodelphinine one or two methoxyl groups were replaced by Cl and a similar result was obtained with pyro-α-oxodelphinine. With each of these derivatives nitric acid was found to remove one *O*-methyl group readily but without substitution. As previously reported, such partial demethylation did not appear to occur so readily with delphinine itself or with the other derivatives tried.

In more recent work, to be described elsewhere (2), in which the Clemmensen reduction was attempted with a keto acid obtained from dihydroisopyrooxodelphonine (3), it was observed that concentration of the reaction mixture resulted in partial demethylation. This effect was then directly traced to the zinc chloride. It was soon found that a very useful reagent is a saturated zinc chloride solution in 5 per cent aqueous HCl. For general orientation the effect of this reagent at 40° was tried on octahydroisopyrooxodelphinine. The crystalline product isolated proved to be a *dimethylanhydro derivative* with the formulation $C_{29}H_{41}O_7N$ and resulted from the loss of two methyl groups and 1 mole of water. The ultraviolet absorption data obtained with this substance, as in the case of isopyrooxodelphonine and dihydroisopyrooxodelphonine as presented in Fig. 1, show essentially end-absorption. These curves are also practically superimposable on the absorption curve previously obtained (4) with α-oxodelphonine, the saponification product of α-oxodelphinine. It is apparent therefore that the double bond produced by the loss of acetic acid on pyrolysis, as well as its subsequent shift by isomerization, has not ap-

preciably affected the absorption. Attempts to hydrogenate the dimethylanhydro derivative were unsuccessful, a fact which, taken together with a similar experience with other derivatives, indicates the formation either of a resistant double bond or of an oxidic group. An oxidic group is favored by the Zerewitinoff determination, which showed the presence of only one active hydrogen due to the remaining free hydroxyl group. The loss of water appears restricted to the newly exposed hydroxyl groups and does not involve the free hydroxyl group present in octahydroisopyrooxodelphinine itself. This point will be discussed again in a succeeding

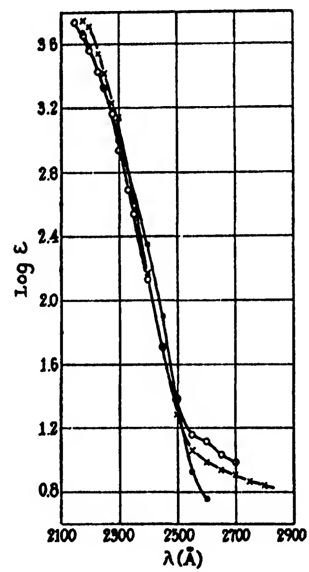


FIG. 1. Ultraviolet absorption spectra of (×) isopyrooxodelphonine, (●) dihydroisopyrooxodelphonine, and (○) the dimethylanhydro derivative in ethanol.

paper. No other demethylation products were isolated from the reaction mixture or when the reaction was tried at higher temperatures. This was in part due to the ready cleavage of the hexahydrobenzoyl group. The apparent greater resistance of the remaining methoxyl groups in octahydroisopyrooxodelphinine is suggested by the following experience with the unsaturated isopyrooxodelphinine in which a labilizing effect of the double bond is indicated.

When the reagent was tried with isopyrooxodelphinine (3) or, better, directly with pyro-α-oxodelphinine because of its initial isomerization to the former by the HCl, demethylation proceeded with unusual ease. At 40° the major reaction involved the loss of three of the *O*-methyl groups

and 1 mole of water. The analytical data showed the formation of the *monomethylanhydro derivative*, $C_{28}H_{31}O_7N$. The latter on hydrogenation absorbed 4 moles of H_2 with the formation of its *octahydro derivative*, $C_{28}H_{39}O_7N$. This reaction involved hydrogenation not only of the benzoyl group to hexahydrobenzoyl but of the pyro double bond, which will be discussed below. That an unsaturation, or preferably an oxidic linkage, produced by the water loss is not the point of hydrogenation is in accord with the result obtained with the demethylation product from octahydroisopyrooxodelphinine.

With the production of the monomethyl compound some complete demethylation was also noted. Further study, however, showed that this was favored at 60° with the production of *desmethylanhydroisopyrooxodelphinine*, $C_{27}H_{29}O_7N$. Because of its solubility long continued extraction with chloroform from the diluted reaction mixture was found necessary for its isolation.

A small quantity of the above intermediate monomethyl derivative was also obtained from the reaction at 60° and in addition a crystalline by-product in still smaller amount, which proved to be a chlorine-containing but completely demethylated derivative. Analysis of this *chlorodesmethylanhydro derivative* indicated a formulation $C_{27}H_{28}O_6NCl$ to be explained by the probable replacement of a hydroxyl group by chlorine.

In a previous communication (1) the production from pyro-α-oxodelphinine with HNO_3 of a substance $C_{30}H_{37}O_8N$ was described with an indicated loss of one methyl group. Because of the ready isomerizing action of the strong acid it is unquestionably an isopyro derivative. That its formation does not involve oxidation by the nitric acid used and represents an intermediate step in the demethylation series has now been shown by its complete demethylation with zinc chloride and HCl to the above desmethylanhydroisopyrooxodelphinine.

Although desmethylanhydroisopyrooxodelphinine was readily hydrogenated, the resulting octahydro derivative possessed properties which made manipulation difficult. Its study was not completed, since the experience with the saponification product proved more satisfactory, as described below. Its oxidation with chromic acid yielded a *monoketone*, $C_{27}H_{27}O_7N$, which was characterized by its *oxime*. The last to be removed and therefore the most resistant methyl group covers a secondary hydroxyl group, since the intermediate monomethylanhydro derivative, $C_{28}H_{31}O_7N$, as well as octahydroisopyrooxodelphinine, was not similarly affected by the reagent.

The desmethylanhydroisopyrooxodelphinine on saponification readily yielded *desmethylanhydroisopyrooxodelphonine*, $C_{20}H_{25}O_6N$, which proved to be soluble in water. On hydrogenation 1 mole of H_2 was absorbed with

the formation of *desmethylanhydrodihydroisopyrooxodelphonine*, $C_{20}H_{27}O_6N$. Its oxidation will be described elsewhere.

Because of the need for larger amounts of pyro-α-oxodelphinine we have studied again its preparation and purification. As presented under "Experimental," chromatography was found necessary for its satisfactory separation from by-products. The presence of a double bond in the pyro derivative was shown (3) by its hydrogenation to octahydropyro-α-oxodelphinine and by the production of an isomeric octahydroisopyro-oxodelphinine from isopyrooxodelphinine. This was supported by the fact that saponification of isopyrooxodelphinine and its octahydro derivative gave respectively isopyrooxodelphonine, $C_{24}H_{35}O_7N$, and its dihydro derivative, $C_{24}H_{37}O_7N$. This would indicate that the isomerization of pyro-α-oxodelphinine with acid is due to a shift of the double bond. More recently this conclusion has been substantiated by the failure of attempts to isomerize its hydrogenation product, octahydropyro-α-oxodelphinine, and also in the following manner.

The previously described hydrogenation product of α-oxodelphinine, hexahydro-α-oxodelphinine (5), was pyrolyzed with loss of acetic acid to *hexahydropyro-α-oxodelphinine* ($[\alpha]_D^{31} = +209°$). As expected, the latter on hydrogenation absorbed the additional mole of H_2 with the formation of the same octahydropyrooxodelphinine ($[\alpha]_D^{31} = -14.5°$), previously obtained by the one-step hydrogenation of pyro-α-oxodelphinine. The rotation found with octahydroisopyrooxodelphinine was $[\alpha]_D^{28} = -72°$.

EXPERIMENTAL

Pyro-α-oxodelphinine—For the preparation of larger quantities of this substance it was found advisable to restrict the amount of α-oxodelphinine used in each pyrolysis operation and then to combine the crude material for the isolation process.

6 gm. portions contained in 200 cc. flasks were immersed in a metal bath heated above 200° and then rapidly brought to 235–240°. The rapid, complete, and uniform melting of the substance was facilitated by the use of a spatula and the heating was continued at this temperature for a total of 12 to 15 minutes, during which complete crystallization of the melt occurred. The product from 42 gm. of starting material was dissolved in chloroform and the solution was extracted successively with dilute Na_2CO_3 and H_2O and dried. After concentration and final drying *in vacuo*, the resinous residue amounted to 33.8 gm. This material was dissolved in 400 cc. of benzene by warming and added to a column of 1 kilo of an active alumina and followed at first by 500 cc. of benzene.

Since the development of the column proceeded too slowly with 1 per cent methanol in benzene, 2 per cent was later used. After several liters

of the 2 per cent mixture had passed through, material suddenly emerged from the column. At this point the first 200 cc. yielded 2 gm. of residue and succeeding 100 cc. portions of eluent yielded in turn 1.95 gm., 2.15, 2.42, 2.62, 2.87, 3.0, 3.04, 3.5, 2.8, and 2.0 gm. Following the succeeding portions of 1.69 and 1.59 gm. of less pure material, the amount eluted dropped rapidly to 0.28, 0.12, 0.08, 0.06 gm., etc. The first eleven fractions of 28.35 gm. were combined by solution in benzene and concentrated to smaller volume and crystallization. A heavy crust of stout prisms separated which were collected with benzene. This fraction of 17.65 gm. was succeeded on concentration by a second of equally pure material of 6.54 gm. The mother liquor yielded more substance but this was contaminated by apparently unpyrolyzed α-oxodelphinine. The substance separated with or without solvent, depending upon the conditions of crystallization. In the former case, it melted at 260–262°. When crystallized from a mixture of benzene and ether, it melted gradually from 246–258°.

$$[\alpha]_D^{20} = +173° \ (c = 0.73 \text{ in } 95\% \text{ ethanol})$$
$$[\alpha]_D^{20} = +88° \ (c = 0.79 \text{ in pyridine})$$

$C_{31}H_{39}O_8N$. Calculated, C 67.23, H 7.10; found, C 67.22, H 7.15

Hexahydropyro-α-oxodelphinine—Hexahydro-α-oxodelphinine was obtained as previously described (5) and, when crystallized from acetone, formed nearly rectangular micro platelets which melted at 194°.

0.4 gm. of this substance was immersed in a metal bath at 200°, the temperature quickly raised to 230°, and the heating continued for 10 minutes. Although the resulting resin crystallized readily from ether, the product was contaminated with starting material and the isopyro derivative. A satisfactory separation was obtained with alumina. A solution of the material in warm benzene was concentrated to about 5 cc. and passed through 10 gm. of an active alumina. After attempted elution with about 70 cc. of benzene, this was followed with 0.5 per cent methanol in benzene, which was collected in 10 cc. portions. Of the latter, Fractions 16, 17, and 18 showed a sharp zone and gave respectively 62, 94, and 71 mg. of crystalline residue. The combined material yielded from ether 0.155 gm. of flat needles and thin leaflets, which melted under the microscope at 212–216° and again gradually partly crystallized as needles which melted at 233–242°.

$$[\alpha]_D^{21} = +209° \ (c = 0.85 \text{ in } 95\% \text{ ethanol})$$

$C_{31}H_{45}O_8N$. Calculated, C 66.50, H 8.11; found, C 66.75, H 8.20

Octahydropyro-α-oxodelphinine—0.1 gm. of the hexahydro derivative was hydrogenated with 50 mg. of platinum oxide catalyst in 95 per cent ethanol. Within an hour the process was practically completed, with the absorption of about 1 mole of H_2 in excess of the catalyst. The substance formed

6-sided leaflets and flat needles from ether which melted at 186–189° and showed no depression when mixed with the previously described (3) octahydro derivative obtained by hydrogenation of pyro-α-oxodelphinine.

$$[\alpha]_D^{31} = -14.5° \text{ (} c = 0.89 \text{ in } 95\% \text{ ethanol)}$$

$C_{31}H_{47}O_8N$. Calculated, C 66.26, H 8.44; found, C 66.42, H 8.55

The hydrogenation of pyro-α-oxodelphinine originally reported in acetic acid solution has since been carried out in ethanol. The resulting octahydro derivative showed $[\alpha]_D^{29} = -14°$ ($c = 0.86$ in 95 per cent ethanol). The rotation obtained with the substance prepared in acetic acid was $[\alpha]_D^{30} = -14°$ ($c = 0.86$ in 95 per cent ethanol). However, the melting point has been found to vary with the conditions of crystallization. On occasion a melting point of 199–204° after preliminary sintering has been observed.

Attempts to isomerize this substance in methanol with HCl, as in the formation of isopyrooxodelphinine (3) from pyrooxodelphinine, failed since aside from slight cleavage of hexahydrobenzoic acid it was recovered unchanged. $[\alpha]_D^{28} = -13°$.

The rotation recently found with octahydroisopyrooxodelphinine is

$$[\alpha]_D^{28} = -72° \text{ (} c = 0.93 \text{ in } 95\% \text{ ethanol)}$$

Dimethylanhydro Derivative from Octahydroisopyrooxodelphinine—0.1 gm. of octahydroisopyrooxodelphinine was treated with a solution of 4 gm. of zinc chloride in 1.4 cc. of 5 per cent HCl and the mixture was kept at 40°. Solution gradually occurred, and after 45 minutes the colorless diluted mixture was continuously extracted for several hours with chloroform. The latter yielded on concentration a resin which smelled of hexahydrobenzoic acid, but readily crystallized on solution in warm methanol-water (2:3). 29 mg. were collected in the first fraction but an additional 7 mg. were obtained in a second fraction. It formed trapezoidal micro platelets or rods from dilute methanol, which melted at 271–275°. Attempts to hydrogenate this substance were unsuccessful.

$C_{29}H_{41}O_7N$.	Calculated, C 67.53,	H 8.02,	2(OCH_3) 12.04
	Found, " 67.77,	" 8.10,	" 11.75

11.493 mg. of dried substance gave 0.58 cc. of CH_4 (30°; 730 mm.).

Found, H 0.197; calculated for 1 H, 0.194

Monomethylanhydro Derivative from Isopyrooxodelphinine—1.65 gm. of pyrooxodelphinine were added to a solution of 66 gm. of zinc chloride in 23 cc. of 5 per cent HCl. The mixture which was kept at 40° formed at first a suspension of partly resinous material, which on stirring gradually dissolved after about 10 minutes, with the exception of a few masses

which had clumped together but on longer manipulation also dissolved. After 1 hour the slightly colored solution was chilled and diluted. The almost clear mixture was extracted continuously in an extractor with chloroform for 1¾ hours. The extract on concentration, finally *in vacuo*, to dryness yielded 1.48 gm. of a resinous mass. On solution in a small volume of acetone and seeding, it gradually crystallized as a mass of micro needles or long platelets. 1.01 gm. were collected in the cold with acetone. From dilute acetone it formed micro prisms and rods which melted at 167–170°. Both from acetone and the diluted solvent it separated with solvent and for analysis was dried at 110° and 0.2 mm.

$[\alpha]_D^{27} = -52°$ (c = 0.90 of dried substance in 50% ethanol)
$C_{28}H_{31}O_7N$. Calculated, C 68.12, H 6.33; found C 68.16, H 6.25

The concentrated mother liquor from the first crystalline crop, when combined with additional material obtained on continued extraction of the original reaction mixture, gradually crystallized as needles of the completely demethylated substance described below.

In the first demethylation experiments isopyrooxodelphinine was used as the starting material and its complete homogeneity was perhaps somewhat in question. It was found necessary in the initial encounter to fractionate the reaction product.

Thus 1 gm. of isopyrooxodelphinine was treated with zinc chloride in 5 per cent HCl, as described above. The resinous reaction product obtained by continuous extraction with chloroform and concentration was dissolved in a few cc. of methanol. The solution was treated with H_2O as long as a resinous precipitate formed. After standing, the decanted solution (about 20 cc.) was allowed to stand. The next day crystallization had begun and, after rubbing and standing overnight, increased. In the meantime the resinous fraction was redissolved in a small volume of acetone and diluted. After standing, the supernatant solution was decanted and seeded with the above fraction. In this manner a small additional fraction was obtained which was collected with the first crystalline crop and amounted in all to 0.137 gm. On recrystallization from dilute acetone it yielded a first crop (55 mg.) which was contaminated by halogen-containing material. The mother liquor on further manipulation gave a second fraction of needles and platelets (38 mg.) which melted at 168–170° and was halogen-free. It was dried for analysis at 110° and 0.2 mm.

$C_{28}H_{31}O_7N$.	Calculated,	C 68.12,	H 6.33,	OCH_3 6.29
	Found,	" 67.84,	" 6.22,	" 6.56

The major amount of the reaction material remained in the resinous precipitate and the mother liquors, and this was recombined by chloroform ex-

traction. The material recovered on concentration to a resin was dissolved in a mixture of 5 cc. of chloroform and 5 cc. of benzene and chromatographed through 20 gm. of alumina in benzene. Since the attempt to develop the column with benzene and then with 0.5 per cent methanol in benzene was without result, it was eluted with 1 per cent methanol in benzene. A succession of fractions was obtained and it was found that the first portion eluted contained halogenated substance. The later intermediate and major band crystallized from the diluted acetone solution as characteristic needles and agreed in properties with the fraction described.

$[\alpha]_D^{27} = -52°$ (c = 0.847 of dried substance in 50% ethanol)
Analysis of Dried Substance—Found, C 67.95, H 6.32, OCH_3 6.40

Later fractions from the chromatogram with 2.5 per cent methanol yielded high melting material which was not further studied.

Octahydromonomethylanhydro Derivative—0.15 gm. of the previous monomethylanhydro derivative was boiled down in methanol to remove acetone of crystallization and the solution in methanol was hydrogenated with 50 mg. of platinum oxide catalyst. Although the process appeared complete after 2 hours, the operation was continued without appreciable further absorption. The absorption in excess of the catalyst requirement was 28 cc., or about 4 moles. After considerable manipulation the substance was obtained from methanol-ether as delicate micro needles or rosettes which melted at 146–147.5° and contained solvent.

For analysis it was dried at 110° and 0.2 mm.

$C_{28}H_{39}O_7N$. Calculated, C 67.02, H 7.84; found, C 66.90, H 7.73

Desmethylanhydroisopyrooxodelphinine—0.1 gm. of the above monomethyl derivative was treated with a solution of 4 gm. of zinc chloride in 1.4 cc. of 5 per cent HCl and heated at 60° for 1 hour. The initial cheesy mass gradually dissolved on manipulation with a rod. The slightly colored solution was diluted and continuously extracted with chloroform. The latter yielded on concentration a gelatinous mass which was further dried *in vacuo*. A solution of the residue in acetone readily crystallized as a mass of delicate needles which melted at 299–301° and contained solvent. Analysis showed it to be methoxyl-free.

For analysis it was dried at 110° and 0.2 mm.

$C_{27}H_{29}O_7N$. Calculated, C 67.61, H 6.10; found, C 67.72, H 6.31

This substance was readily obtained in a larger amount by the direct use of pyrooxodelphinine. 2 gm. of the latter, when heated at 60° in

a solution of 80 gm. of zinc chloride in 28 cc. of 5 per cent HCl, gradually dissolved. After 1 hour the clear yellow solution was diluted and extracted in a funnel four times with 40 cc. portions of chloroform. The latter yielded on concentration 0.49 gm. of a mixture. The aqueous phase was then continuously extracted with chloroform for 18 hours, during which partly gelatinous material separated in the lower flask of accumulated boiling solvent. The latter on concentration, finally *in vacuo*, to dryness yielded 1.3 gm. of partly crystalline material. This was gradually dissolved by refluxing in a large volume (about 700 cc.) of acetone, and, on concentration to about 200 cc., some gelatinous material began to appear but was soon followed by delicate needles. After standing overnight the solid was collected with dry acetone. 0.76 gm. of substance which melted at 299–300° was obtained.

$$[\alpha]_D^{28} = -48.5° \text{ (c = 0.89 of dried substance in 50\% ethanol)}$$

Found, C 67.22, H 6.24

Material obtained by continued extraction of the aqueous phase and the above acetone mother liquor yielded an additional amount of the same substance and also a fraction of the intermediate monomethyl derivative.

The 0.49 gm. fraction of more readily extracted material, when dissolved in a small volume of chloroform, deposited leaflets which were collected with chloroform. The substance gradually softened to a melt from 240–249° and contained chlorine. The methoxyl content proved negligible.

For analysis the substance was dried at 110° and 0.2 mm.

$C_{27}H_{28}O_6NCl$.	Calculated.	C 65.10, H 5.67, Cl 7.12
	Found.	" 64.42, " 5.94, " 6.92

0.2 gm. of the substance $C_{30}H_{37}O_8N$ obtained from pyro-α-oxodelphinine with HNO_3 (1) was similarly heated for 45 minutes at 60° with zinc chloride solution. The somewhat colored solution on dilution caused some precipitation of resin and was shaken out four times with about 50 cc. of chloroform. The dried extract on concentration yielded 0.11 gm. of a resin. This consisted mostly of intermediate demethylation products and halogen-containing material and was not further investigated.

The remaining aqueous phase was continuously extracted with chloroform for 20 hours and yielded partly gelatinous material which, when dried, weighed 66 mg. This yielded from acetone a first crop of 41 mg. of delicate needles which melted at 295–296.5°. Analysis of the dried substance gave

$C_{27}H_{29}O_7N$. Calculated, C 67.61, H 6.10; found, C 67.20, H 6.09

Desmethylanhydroketone, $C_{27}H_{27}O_7N$—0.15 gm. of desmethylanhydroisopyrooxodelphinine was dissolved in a mixture of 2.3 cc. of acetic acid and 0.75 cc. of H_2O and treated with 0.3 cc. of the Kiliani CrO_3 solution. Oxidation gradually proceeded at room temperature and the reagent was used up in about 1.5 hours. The diluted mixture, after continuous extraction with chloroform, yielded 0.14 gm. of resin. It crystallized from acetone as long thin needles which contained solvent and melted at 191–194°. It is appreciably soluble in water.

For analysis it was dried at 110° and 0.2 mm.

$C_{27}H_{27}O_7N$. Calculated, C 67.89, H 5.70; found, C 67.76, H 6.02

The *oxime* was prepared by allowing the substance to react with a mixture of hydroxylamine hydrochloride and sodium acetate in sufficient 50 per cent methanol. After several days standing and the removal of methanol, material separated which was not definitely crystalline. On dilution of the warm solution in ethanol it separated as a fine micro crystalline powder which melted at 245–248°. The anhydrous substance yielded

$C_{27}H_{28}O_7N_2$.	Calculated, C 65.82, H 5.73, N 5.69
	Found, " 65.82, " 6.18 " 5.38

Desmethylanhydroisopyrooxodelphonine—1 gm. of desmethylisopyrooxodelphinine was suspended in 10 cc. of 70 per cent ethanol and treated with 4 cc. of 0.5 N NaOH. The substance gradually dissolved but there remained in suspension some apparently amorphous material. After 50 minutes the turbid mixture was somewhat diluted and carefully acidified to Congo red with H_2SO_4. After repeated extraction with ether to remove benzoic acid and ester, the aqueous phase was treated with several volumes of absolute ethanol and filtered. The filtrate was carefully neutralized with dilute NaOH to phenolphthalein and concentrated *in vacuo* to dryness. The residue was extracted with absolute ethanol and filtered from Na_2SO_4. The filtrate on concentration gave 0.8 gm. of residue. On treating with acetone-water (4:1) it dissolved on warming and crystallized on seeding as a thick pap. The first fraction collected with the same solvent was 0.28 gm. The mother liquor yielded successive fractions of 0.19 gm., 40 mg., and 22 mg. It formed needles which contained a solvent and melted at 168–170°. It was difficult to obtain the substance entirely ash-free. Attempts to sublime it at 0.03 mm. were unsuccessful, except for a relatively very small amount which flashed over apparently with solvent with the bath at about 120°. This substance melted at 171–173°. When the bath was further heated to 210°, decomposition in the unsublimed residue occurred. Molecular sublimation was

not tried. The substance is readily soluble in H_2O, methanol, and ethanol, and insoluble in ether.

For analysis it was dried at 110° and 0.2 mm.

$C_{20}H_{25}O_6N$.	Calculated.		C 63.96, H 6.72
	Found.	(*a*)	" 63.57, " 6.73
		(*b*)	" 63.76, " 6.83

Desmethylanhydrodihydroisopyrooxodelphonine—0.1 gm. of desmethylanhydroisopyrooxodelphonine in 50 per cent ethanol with 50 mg. of platinum oxide catalyst absorbed about 1 mole of H_2 in excess of the catalyst requirement within 25 minutes and no further absorption appeared to occur. The filtrate from the catalyst on concentration to dryness yielded a resin which was dissolved in acetone-water (9:1). When crystallization once began after long standing, it separated readily as a powder of glistening minute micro crystals which were solvent-free. It crystallized also from methanol in a similar form. The substance melts at 290–292° and is soluble in H_2O but very sparingly in absolute ethanol, methanol, and acetone.

$C_{20}H_{27}O_6N$.	Calculated.		C 63.62, H 7.21
	Found.	(*a*)	" 63.34, " 6.92
		(*b*)	" 63.93, " 7.31

The yield of this product was somewhat less than half of the starting material. The mother liquor contained an appreciable amount of more soluble, possibly stereoisomeric, substance which was not crystallized.

All analytical data have been obtained by Mr. D. Rigakos of this laboratory.

SUMMARY

Although instances of partial demethylation of the methoxyl groups of certain delphinine derivatives were previously noted, controlled partial and complete demethylation has now been accomplished with the unsaturated isopyrooxodelphinine. This readily yields with zinc chloride and HCl a monomethyl- or a desmethylanhydro derivative with an accompanying loss of 1 mole of water. In the case of the hydrogenated derivatives, demethylation occurs most readily to the dimethyl stage, with an accompanying loss of 1 mole of water. The last and most resistant methoxyl group involves a secondary hydroxyl which can be oxidized to carbonyl after demethylation.

The isomerization of pyro-α-oxodelphinine to isopyrooxodelphinine has been shown to involve the double bond produced on pyrolysis of α-oxodelphinine with loss of acetic acid.

BIBLIOGRAPHY

1. Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **136**, 303 (1940).
2. Jacobs, W. A., and Sato, Y., *J. Biol. Chem.*, in press.
3. Jacobs, W. A., and Huebner, C. F., *J. Biol. Chem.*, **170**, 209 (1947).
4. Craig, L. C., Michaelis, L., Granick, S., and Jacobs, W. A., *J. Biol. Chem.*, **154**, 293 (1944).
5. Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **128**, 431 (1939).

[Reprinted from the JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, April, 1949, Vol 71, No. 4, pp. 1146–1149]

THE ELECTROPHORETIC PROPERTIES OF PLAKALBUMIN

BY GERTRUDE E. PERLMANN

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, December 2, 1948)

INTRODUCTION

In a recent note, Linderstrøm-Lang and Ottesen[1] showed that ovalbumin is transformed, by the action of an enzyme from *Bacillus subtilis*, into a protein that crystallizes as rectangular plates from ammonium sulfate. They named this protein plakalbumin.[2] Since the transformation of ovalbumin to plakalbumin, A→P, occurs without serious degradation of the protein,[1] it seemed of interest to compare the electrophoretic behavior of the two materials. Consequently, the preliminary experiments[3] that indicated that plakalbumin differed electrophoretically from its parent substance, ovalbumin, have been extended and the new results are reported below.

EXPERIMENTAL

Plakalbumin was obtained by incubating a 6% solution of thrice recrystallized ovalbumin[4] in 0.02 ionic strength phosphate buffer at *p*H 6.8 with an enzyme preparation from *B. subtilis*, kindly supplied by Linderstrøm-Lang. After incubation for six hours at 30°, the solution was acidified with hydrochloric acid to *p*H 5.4 and the protein crystallized as rectangular plates by the addition of saturated ammonium sulfate solution.[1]

The electrophoretic experiments were carried out at 0.5° in the apparatus described by Longsworth.[5, 6] Unless noted otherwise below, a protein concentration of 1% was used in the experiments and prior to electrophoresis the protein solutions were dialyzed for two to three days against liberal portions of an appropriate buffer. The mobilities were computed from the descending patterns and refer to 0°.

RESULTS

In Fig. 1 are superimposed the tracings of the patterns obtained after electrophoresis of ovalbumin and plakalbumin in a 0.1 ionic strength phosphate buffer at *p*H 6.8 at a potential gradient of 6 volts per cm. for 9900 seconds. Under these conditions superposition of the patterns permits not only a direct

(1) Linderstrøm-Lang and Ottesen, *Nature*, **159**, 807 (1947).
(2) Eeg-Larsen, Linderstrøm-Lang and Ottesen, *Arch. Biochem.*, **19**, 340 (1948).
(3) Perlmann, *Nature*, **161**, 720 (1948).
(4) Sørensen and Høyrup, *Compt. rend. Lab. Carlsberg*, **12**, 12 (1917).
(5) Longsworth, *Chem. Reviews*, **30**, 323 (1942).
(6) Longsworth, *Ind. Eng. Chem., Anal. Ed.*, **18**, 219 (1946).

comparison of the relative concentrations of the components but also of their approximate mobilities. It is thus clear from Fig. 1 that although the patterns are similar in appearance the plakalbumin has a lower mobility, u, than its parent substance ovalbumin. The actual values are -5.9×10^{-5} and -5.5×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} for the main components, A_1 and P_1, of ovalbumin and plakalbumin, respectively, and -4.8×10^{-5} and -4.3×10^{-5} for the components A_2 and P_2. Plakalbumin from a sample of ovalbumin incubated for twenty-four hours gave the same results. Furthermore, as is the case with ovalbumin[7] at *p*H values below 4.0, plakalbumin also migrates over a distance of at least 7.0 cm. in the channel of the electrophoresis cell without resolution of the boundary into more than one peak.

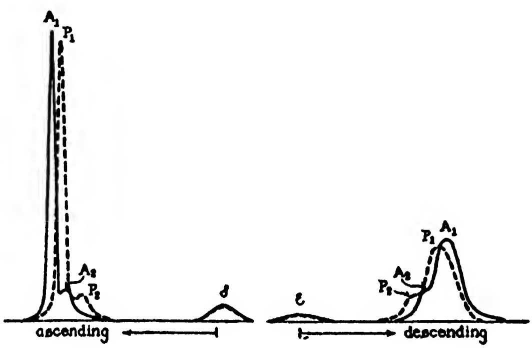


FIG. 1. Superimposed tracings of electrophoretic patterns of a 1% solution of ovalbumin,—, and plakalbumin, - - -, after electrophoresis in sodium phosphate buffer at *p*H 6.8 and 0.1 ionic strength for 9900 seconds at 6 volts per cm.

In the preliminary work on plakalbumin it was observed that the relative concentration of the components, P_1 and P_2, but not their mobilities, changed on repeated crystallization of the freshly prepared protein. However, if the time between successive crystallizations is kept at a minimum and if the plakalbumin is stored as a paste under saturated ammonium sulfate, no significant alteration occurs. On the other hand, prolonged dialysis, or storage of plakalbumin in solution, causes marked changes. Thus, in a solution stored at 3° for ten days, the amount of the slowly moving component, P_2, had increased from 16 to 44% at the expense of the faster one, P_1. This result may be considered in connection with the observation of the Danish workers that in plakalbumin solutions at *p*H values above 5, even after repeated recrystallizations and dialysis a slow liberation of non-protein nitrogen occurs.[2]

Mobilities of Ovalbumin and Plakalbumin at Different pH Values.—Much of this work has been directed toward the determination of the mobilities of

(7) Longsworth, Cannan and MacInnes, THIS JOURNAL, **62**, 2580 (1940).

the two proteins at various *p*H values. The results of these measurements are presented in Table I, in which the nature and composition of the buffers are listed in the first column while the *p*H of the protein solution in equilibrium therewith is given in column 2. The mobilities listed in this table refer in each case to the fast moving component of the proteins, except for those at *p*H values below 4.0 where the proteins migrate as a single peak. In these cases the

TABLE I

Mobilities of Plakalbumin and Ovalbumin in Buffer Solutions of Ionic Strength 0.1

Ac = acetate; Cac = cacodylate; V = diethyl barbiturate

1	2	3 $u \times 10^5$	4	5
Buffer	PH, 25°	Plakalbumin	Ovalbumin	Δu
0.1 *N* HCl—0.5 *N* glycine	3.10	6.54	5.95	0.59
.1 *N* NaAc—0.5 *N* HAc	3.91	3.25	2.71	.54
.1 *N* NaAc—0.14 *N* HAc	4.50	1.06		
.1 *N* NaAc—0.1 *N* HAc	4.64	0.29	−0.25	.54
.1 *N* NaAc—0.06 *N* HAc	4.89	−0.74		
.1 *N* NaAc—0.01 *N* HAc	5.65	−2.91	−3.50	.59
.02 *N* NaCac—0.004 *N* HCac—0.08 *N* NaCl	6.79	−4.50	−5.16	.66
.02 *N* NaV—0.02 *N* HV—0.08 *N* NaCl	7.82	−5.62	−5.65	
.1 *N* NaV—0.02 *N* HV	8.60	−5.84	−5.78	
.1 *N* NaV—0.005 *N* HV	9.10	−5.93	−5.92	

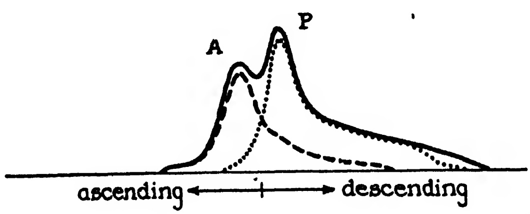


FIG. 2. Superimposed tracings of electrophoretic patterns of a 1.2% mixture of 33% ovalbumin and 67% plakalbumin,—, of a 0.4% solution of ovalbumin, - - -, and of a 0.8% solution of plakalbumin, ... , in 0.1 *N* sodium acetate buffer at *p*H 4.64 at 6 volts per cm. for 28800 seconds.

mobilities are mean mobilities. A comparison of the figures for plakalbumin, column 3, with those for ovalbumin, column 4, shows that the mobilities of the two proteins at any given *p*H below about 7.0 differ by 0.5 to 0.6 $\times$ 10^{-5}, column 5. At *p*H values above *p*H 7.8 the two proteins migrate with the same velocity.

The Behavior of Mixtures of Plakalbumin and Ovalbumin.—Although the mobility differences below *p*H 7.0 are sufficient to lead one to expect resolution

of the ovalbumin and plakalbumin on mixing, attempts in a 0.1 ionic strength phosphate buffer at *p*H 6.8 and in an 0.1 ionic strength hydrochloric acid-glycine buffer at *p*H 3.0 failed. The resulting patterns for the mixture were identical in appearance with those of either protein except that the boundary displacement corresponded to mobility values intermediate between those of the components of the mixture. Resolution is obtained, however, at a *p*H of 4.64, *i.e.*, between their isoelectric *p*H values. This is shown in *Fig. 2* where the

TABLE II

Dependence of the Isoelectric pH on Ionic Strength

1	2	3	4 *p*I Plakalbumin	5 *p*I Ovalbumin	6
μ	*p*H[a]	$u_p \times 10^5$			Δ *p*I
0.1	4.50	1.06			
	4.67	0.29	4.72	4.58	0.14
	4.90	−0.74			
.05	4.68	0.82	4.77	4.63	.14
	4.88	−0.75			
.02	4.73	0.7	4.82	4.68	.14
	4.96	−1.54			
.01	4.68	1.95	4.86	4.71	.15
	4.96	−1.06			

[a] *p*H values refer to 0° by applying a correction of 0.025 to the values measured at 25°.[8]

full line represents the patterns obtained after electrophoresis of a 1.2% solution of a mixture of 33% ovalbumin and 67% plakalbumin at a potential gradient of 6 volts per cm. for 28,800 seconds. The dashed and dotted curves superimposed upon this pattern are those of ovalbumin and plakalbumin, respectively, obtained in separate experiments under identical conditions.

Dependence of the Isoelectric pH of Plakalbumin upon Ionic Strength in Acetate Buffers.—As is shown by the data of Table II the isoelectric *p*H of plakalbumin, designated here as *p*I, shifts to higher values at low ionic strength in acetate buffers. The *p*I values at each ionic strength, column 4, were obtained by linear interpolation of the *p*H mobility values listed in the two preceding columns of this table. Owing to the complexity of the electrophoretic patterns of plakalbumin at *p*H values just below the isoelectric *p*H, the first moment of the entire gradient curve of the descending pattern is used for the computation of the mean mobility, reported in Table II.

For comparison, the *p*I values of ovalbumin obtained by Tiselius and Svensson[8] are listed in column 5 of Table II. Except for a constant difference of 0.14 *p*H unit, it is apparent that the isoelectric *p*H of plakalbumin changes with the buffer salt concentration in the same manner as does that of ovalbumin.

(8) Tiselius and Svensson, *Trans. Faraday Soc.*, **36**, 16 (1940).

The response of plakalbumin to the partial substitution of chloride for acetate in these buffers is also similar to that of ovalbumin; thus if 80% of the acetate ion in a 0.1 *N* buffer is replaced by chloride ion, the isoelectric *p*H of plakalbumin is reduced to 4.58, whereas that of ovalbumin to 4.44, a difference of 0.14 *p*H unit as in the case of the pure acetate buffers, Table II. This displacement of the isoelectric *p*H on substitution of chloride for acetate toward a more acid reaction is comparable with that observed in the case of other proteins.[9]

DISCUSSION

Since there is considerable evidence for a constant proportionality between mobility and the net charge of the protein ion[10, 11] it is of interest to compare the change in mobility Δu, accompanying the A→P transformation, with other properties of these two proteins. Of the many explanations for this change that might be advanced the following is a reasonable one at the present time. It is based, in part, on the observation of Eeg-Larsen, Linderstrøm-Lang and Ottesen[2] that the fragments that are lost by the ovalbumin in the A→P process contain a glutamic and possibly an aspartic acid residue in addition to other amino acid residues that would not have ionizing groups when linked, in the α-position, with peptide bonds in the intact protein molecule. In the following discussion the number of ionizing groups that are lost in the A→P process will simply be designated as a. If, therefore, ovalbumin can be idealized as peptide chain, $(HOOC)_mR(NH_2)_n$ with m carboxyl groups that dissociate in the region from *p*H 2.0 to 6.5 and n amino groups that dissociate above *p*H 8.5, plakalbumin would be $(HOOC)_{m-a}R'(NH_2)_n$. In strongly acid solution the ovalbumin ion would be $(HOOC)_mR(NH_3^+)_n$ with a net charge, z, of $+n$. Under these conditions plakalbumin should have the same net charge as ovalbumin and if the friction coefficients of the two molecules were the same the mobilities should be identical at a given low *p*H. A neutral reaction the ovalbumin ion would be $(^-OOC)_mR(NH_3^+)_n$ with a net charge of $n = m$ and since the mobility is negative at this *p*H one can conclude that $m > n$. Under these conditions the net charge of plakalbumin would be $n - m + a$ and a curve of z against *p*H, as abscissas, for this protein should be above that of ovalbumin except in acids solutions where the two curves approach the common limiting value of $z = n$. If the a carboxyl groups removed in the A→P reaction are strong ones they will lose their protons at *p*H values below the isoelectric zone and ovalbumin will thus have an isoelectric *p*H below that of plakalbumin in which these groups are missing. The experimental observation, Table II, that this is the case thus supports the view that the carboxyl groups that are involved in the A→P transformation are relatively strong ones.

However, the mobility values of plakalbumin approach those of ovalbumin

(9) Longsworth and Jacobsen, *J. Phys. and Coll. Chem.*, **53**, 126 (1949).
(10) Abramson,. *J Gen. Physiol.*, **15**, 575 (1931-1932).
(11) Longsworth, *Ann. N. Y. Acad. Sci.*, **41**, 267 (1941).

at neutrality instead of at acid reactions as the idealized case described above would require. This suggests that the friction coefficients, f, of the two proteins are not identical but that for plakalbumin, f_P is enough lower than that for ovalbumin, f_A, to compensate for the smaller net negative charge at neutrality. At acid reactions where the net charge of both proteins approaches the common value, n, the smaller value of f_P, as compared with f_A, would lead to the higher mobilities for plakalbumin that are observed.

If at any given pH, the values of u_A and u_P refer to the same buffer solvent, the friction coefficients may be defined so that $u_A = z_A/f_A$ and $u_P = z_P/f_P$, where the ratio, f_A/f_P, should be independent of the pH, the ionic strength and the nature of the buffer ions. At the isoelectric pH of plakalbumin, $u_P = z_P = 0$

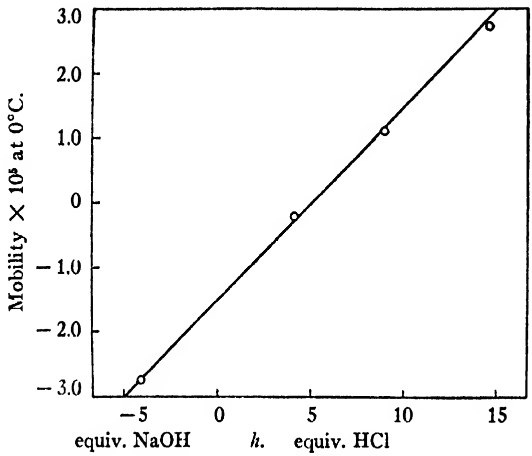


FIG. 3. Plot for the evaluation of the proportionality factor between mobility and titration increment for ovalbumin.

and since the valence of ovalbumin at this pH, *i.e.*, 4.72, is assumed to differ from that of plakalbumin only by the a ionized carboxyl groups that are present in the one protein but missing from the other, $z_A = -a$, and the mobility of ovalbumin, $u_A = -0.6 \times 10^{-5} = a/f_A$ may then be used to estimate the value of a. Here $1/f_A$ may be taken as the slope, 3.0×10^{-6}, of the line that is obtained by plotting[9] as shown in Fig. 3, the mobilities of ovalbumin against the equivalents, h, of acid in alkali bound per mole of protein at an ionic strength 0.1 and at the given pH.[12] Hence $a = 2$, is in agreement with the result by Eeg-Larsen, Linderstrøm-Lang and Ottesen.[2] In view, however, of the arbitrary nature of the assumptions that have been made in the use of the mobility data for the evaluation of a, this agreement should not necessarily be interpreted as confirmation of the chemical analysis. Probably the most

(12) Cannan, Kibrick and Palmer, *Ann. N. Y. Acad. Sci.*, **41**, 243 (1941).

that should be said is that the electrophoretic results are not inconsistent with the analytical ones.

In experiments described elsewhere,[3] the author has also observed that plakalbumin differs electrophoretically from the ovalbumin modification reported by MacPherson, Moore and Longsworth.[13] Unpublished results further indicate that their modification is also a product of an enzymatic reaction. It, therefore, appears possible to transform, without serious degradation, a protein like ovalbumin into several other well-defined proteins which differ from each other in at least one property, namely, in their behavior in the electric field.

Acknowledgment. The author is much indebted to Dr. L. G. Longsworth of these Laboratories for suggestions as to the interpretation of the experimental results and also to him and to Dr. D. A. MacInnes for criticism of the manuscript. My thanks go also to Professor K. Linderstrøm-Lang of the Carlsberg Laboratory, Copenhagen, for his constant stimulating interest in this research.

SUMMARY

The electrophoretic behavior of plakalbumin, a protein derived from ovalbumin by the action of an enzyme from *B. subtilis*, has been compared with that of the parent substance. At *p*H values below about 7.0, the mobilities differ by 0.5 to 0.6 $\times$ 10^{-5} whereas at more alkaline reactions plakalbumin and ovalbumin are indistinguishable electrophoretically. The isoelectric *p*H of plakalbumin at all ionic strengths investigated is 0.14 *p*H unit above that of ovalbumin.

(13) MacPherson, Moore and Longsworth, *J. Biol. Chem.*, **156**, 381 (1944).

[Reprinted from the Journal of the American Chemical Society, April, 1949, Vol 71, No. 4, pp. 1496–1498]

EXTRAPOLATION OF CONDUCTANCE DATA FOR WEAK ELECTROLYTES

BY RAYMOND M. FUOSS AND THEODORE SHEDLOVSKY

(From the Sterling Chemistry Laboratory, New Haven, Connecticut, and the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, February 9, 1949)

Unambiguous extrapolations of conductance data, in order to determine Λ_0, the limiting conductance, and K, the dissociation constant for weak electrolytes, are possible in aqueous systems by synthesizing a hypothetical conductance curve for the completely dissociated portion of the weak electrolyte from conductance data on the corresponding strong electrolytes,[1] *e.g.*

$$\Lambda^*_{(\mathrm{HAc})} = \Lambda_{(\mathrm{HCl})} - \Lambda_{(\mathrm{NaCl})} + \Lambda_{(\mathrm{NaAc})} \tag{1}$$

in which the Λ values refer, of course, to the same ion concentration. The degree of dissociation is then given by Λ/Λ^*, since $\Lambda = 1000\kappa/c$ and $\Lambda^* = 1000\kappa/c_i$, κ being the specific conductance, c the equivalent electrolyte concentration, and c_i the corresponding free ion concentration.

In non-aqueous systems, where the dielectric constant of the solvent is about 40 or less, this procedure cannot be employed since no strong electrolytes exist in such solvents. It therefore becomes necessary to evaluate Λ_0 and K from the conductance data for the electrolyte itself. Two extrapolation methods have been proposed.

Fuoss and Kraus[2] wrote for 1–1 electrolytes

$$\Lambda = \gamma(\Lambda_0 - \alpha\sqrt{c\gamma}) \tag{2}$$

where γ is the degree of dissociation defined as the ratio of the concentration of free ions to the stoichiometric concentration c and α is the Onsager coefficient

$$\alpha = 8.2 \times 10^5\Lambda_0/(DT)^{3/2} + 82/\eta(DT)^{1/2} \tag{3}$$

Where D is the dielectric constant, η the viscosity of the solvent, and T the absolute temperature. Equation (2) was solved for γ in terms of a function[3] $F(z)$ where

$$z = \alpha\sqrt{c\Lambda}/\Lambda_0^{3/2} \tag{4}$$

(1) D. A. MacInnes, This Journal, **48**, 2068 (1926); M. S. Sherrill and A. A. Noyes, *ibid.*, **48**, 1861 (1926); D. A. MacInnes and T. Shedlovsky, *ibid.*, **54**, 1430 (1932).

(2) R. M. Fuoss and C. A. Kraus, *ibid.*, **55**, 476 (1933).

(3) R. M. Fuoss, *ibid.*, **57**, 488 (1935).

and

$$\gamma = \Lambda/\Lambda_0 F(z) \tag{5}$$

Substituting Eq. (5) in the mass action equation and rearranging, one obtains

$$F/\Lambda = 1/\Lambda_0 + c\Lambda f^2/FK_F\Lambda_0^2 \tag{6}$$

where f is the activity coefficient which is computed from $(-\log f) = \beta\sqrt{c_i}$. A plot of F/Λ against $c\Lambda f^2/F$ is linear at low concentrations and extrapolates to the reciprocal of the limiting equivalent conductance, with slope $1/K_F\Lambda_0^2$.

Shedlovsky[4] later proposed the equation

$$\Lambda = \Theta\Lambda_0 - \alpha(\Lambda/\Lambda_0)\sqrt{c\Theta} \tag{7}$$

where Θ is the degree of dissociation. Equation (7) is a quadratic in $\Theta^{1/2}$ and is therefore much simpler to solve than Eq. (2), which is a cubic in $\gamma^{1/2}$. The solution of (7) may then be written as

$$\Theta = S(z)\Lambda/\Lambda_0 \tag{8}$$

where $S(z) \equiv (z/2 + \sqrt{1 + (z/2)^2})^2$, and z is defined by Eq. (4). Substituting in the mass action equation and rearranging, we obtain

$$1/\Lambda S = 1/\Lambda_0 + c\Lambda S f^2/K_S\Lambda_0^2 \tag{9}$$

Here, at low concentrations, $1/\Lambda S$ is linear in $c\Lambda S f^2$, with intercept $1/\Lambda_0$ and slope $1/K_S\Lambda_0^2$. The intercepts of Eqs. (6) and (9) are found to be identical, but the slopes, and therefore the dissociation constants[5] are sometimes different. The question naturally arises as to which of the two values to choose for the dissociation constant in such cases.

The derivation[2] of Eq. (2) is based on the Onsager limiting law

$$\Lambda = \Lambda_0 - \alpha\sqrt{c} \tag{10}$$

If Onsager had computed resistance instead of conductance, the limiting law would have read

$$1/\Lambda = 1/\Lambda_0 + (\alpha/\Lambda_0^2)\sqrt{c} \tag{11}$$

which rearranges to

$$\Lambda = \Lambda_0 - \alpha(\Lambda/\Lambda_0)\sqrt{c} \tag{12}$$

Shedlovsky[4] has shown that Eq. (12) reproduces conductance data to significantly higher concentrations than does Eq. (10). The derivation[4] of Eq. (7) was based on the Onsager limiting law in the form (12), and results based on Eq. (9) are therefore to be preferred.

(4) T. Shedlovsky, *Jour. Franklin Inst.*, **225**, 739 (1938).

(5) H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," Reinhold Publishing Corp., New York, N. Y., 1943; p. 190.

The difference between the various functions is illustrated in Fig. 1. Curve 2 represents actual data for potassium chloride[6] where $\Lambda_0 = 149.9$ and $\alpha = 93.9$. Curve 4 is for the familiar Onsager linear limiting Eq. (10). Curve 3 is for Eq. (12), using the same constants; it will be noted that it gives a closer approximation of the experimental results than Curve 4, *i.e.*, Eq. (10). Data for iodic acid,[7] a somewhat weak electrolyte, for which $K = 0.16$, $\Lambda_0 = 391.2$, $\alpha = 150$ are plotted in Curve 5. Although, strictly speaking, the abscissa, $\sqrt{c_i}$, depends on how c_i is computed, *e.g.*, by the Fuoss or by the Shedlovsky function, these differences are too small to appear in Curve 5 on the scale of the figure. Due to ion association[8] (weak electrolytes) there are found strong negative deviations from the Onsager conductance equation, such as in Curve 5. The curves 2, 3 and 4 for potassium chloride are for $\Lambda/\Lambda_0 = (1000\kappa/c)(1/\Lambda_0)$ *vs.* $\sqrt{c_i}$, but since this salt is a strong electrolyte $c_i = c$. The slope of the linear Curve 4, $(\alpha/\Lambda_0)_{KCl}$ is 0.63, whereas the corresponding value for the ionized portion of iodic acid, $(\alpha/\Lambda_0)_{HIO_3}$ is 0.38. Let us change the ordinate scale for these three curves so that the slope for Curve 4 is 0.38 instead of 0.63. We may then suppose that the curves 2, 3 and 4 refer to $(1000\kappa/c_i)(1/\Lambda_0)$ *vs.* $\sqrt{c_i}$ for iodic acid instead of potassium chloride, and recall that for a weak electrolyte c_i is the product of the stoichiometric concentration and of the degree of dissociation. The latter is therefore obtained as the ratio of $\Lambda/\Lambda_0 = (1000\kappa/c)(1/\Lambda_0)$ to

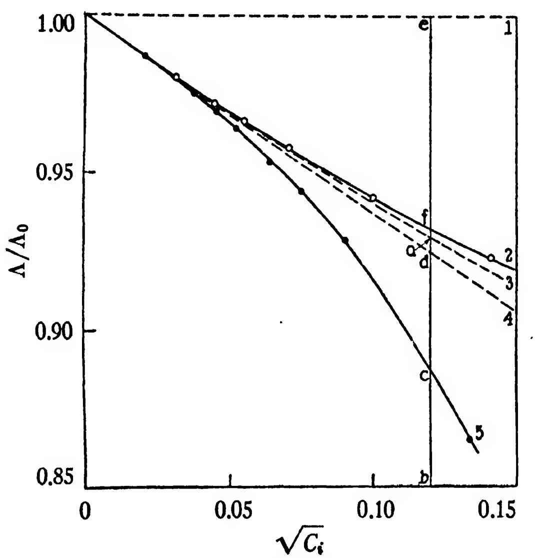


FIG. 1. Comparison of conductance functions

(6) T. Shedlovsky, THIS JOURNAL, **54**, 1411 (1932).
(7) C. A. Kraus and H. C. Parker, *ibid.*, **54**, 2429 (1932).
(8) R. M. Fuoss, *Chem. Revs.*, **17**, 27 (1935).

$(1000\kappa/c_i)1\Lambda_0$ at the same value of $\sqrt{c_i}$. Thus, the Fuoss function assumes that *cb/db* is a measure of the degree of dissociation, while the Shedlovsky function uses the ratio *cb/ab*, which is a better approximation, and incidentally the one which would naturally have been used if the limiting Onsager equation had been originally presented in the form of Eq. (11). The synthetic method (Eq. (1)) uses the ratio *cb/fb* but as we have already pointed out, it is not available in solvents with dielectric constants below 40.

Extrapolation by Eqs. (6) or (9) is essentially use of the Ostwald dilution law, corrected for the effect of long range interionic forces on mobility and thermodynamic potential; the degree of dissociation in the classical theory, Λ/Λ_0, is measured by the ratio *cb/eb* of Fig. 1.

The difference between K_S and K_F can be found as follows. By equating the two expressions, Eqs. (2) and (7) for Λ, substituting Eqs. (5) and (8) and solving, we obtain

$$\gamma = \Theta[1 + z(F^{-3/2}S^{-1} - S^{-1/2})] \tag{13}$$

$$\equiv \Theta(1 + x) \tag{13'}$$

If we now substitute (13) in the mass action Eq. (6) and rearrange, we obtain

$$1/\Lambda S = 1/\Lambda_0 + c\Lambda S f^2(1 + x)^2/K_F\Lambda_0^2 + x/\Lambda_0 \tag{14}$$

Expansion of $S(z)$ and $F(z)$ in their power series gives

$$x = z^2 + O(c^{3/2}) \tag{15}$$

and at low concentrations Eq. (14) thus reduces to

$$1/\Lambda S = 1/\Lambda_0 + c\Lambda S f^2/K_F\Lambda_0^2 + \alpha^2 c\Lambda/\Lambda_0^4 \tag{16}$$

The limiting slope of Eq. (16) is

$$\frac{d(1/\Lambda S)}{d(c\Lambda Sf^2)_{c=0}} = \frac{1}{K_F\Lambda_0^2} + \frac{\alpha^2}{\Lambda_0^4} = \frac{1}{K_S\Lambda_0^2} \tag{17}$$

whence

$$1/K_S = 1/K_F + \alpha^2/\Lambda_0^2 \tag{18}$$

The difference shown in Eq. (18) is a consequence of the *difference* between Θ and γ; in the limit of zero concentration, both Θ and γ approach unity, but as is readily seen from Eqs. (13′) and (15), their difference is linear in concentration, and the mass action extrapolation is essentially one on a linear concentration scale. According to Eq. (3), the ratio α/Λ_0 has the following upper bounds if we set the Walden product $\Lambda_0\eta = 0.5$: $D = 40, \alpha/\Lambda_0 \leqslant 2$; $D = 20, \alpha/\Lambda_0 \leqslant 4$; $D = 10, \alpha/\Lambda_0 \leqslant 8$. Since K decreases[9] rapidly as D decreases, the two methods of extrapolation will not differ significantly when K is less than about 10^{-3}. In the approximate range $10^{-3} \leqslant K \leqslant 1$, however, we recommend extrapolation by means of Eq. (9).

(9) R. M. Fuoss and C. A. Kraus, This Journal, **55**, 1019 (1933).

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, May, 1949,
Vol. 179, No. 1, p. 505]

THE PHEOPORPHYRIN NATURE OF CHLOROPHYLL *c*

BY S. GRANICK

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, March 11, 1949)

Strain and coworkers[1] have definitely established the fact that there exists in the diatoms, dinoflagellates, and brown algae, besides chlorophyll *a*, another green pigment to which they have given the name *chlorophyll c*. We have recently isolated a small quantity of this pigment from a *Laminaria* species obtained from Woods Hole, and have confirmed the absorption spectrum of Strain *et al.*, which so far has been the only property identifying this pigment.

We have now found that this compound is a Mg complex, the Mg having been identified by a micro modification of the titan yellow method.[2] In contrast to the traces of acid which are sufficient to split Mg from chlorophyll *a* and *b*, the removal of Mg from this compound requires a surprisingly high acidity, in the neighborhood of 3 to 4 N HCl.

The HCl number of this compound devoid of Mg is about 12, indicating that it does not possess a phytol group. Its spectrum resembles that of a pheoporphyrin rather than a pheophorbide (*i.e.* pyrrole Ring IV is not reduced). The presence of a cyclopentanone ring is suggested by the positive phase test and the formation of a chloroporphyrin type of spectrum on treatment with methyl alcoholic HCl.

These properties of chlorophyll *c* suggest that this compound may be a modified Mg pheoporphyrin, containing an as yet unidentified chromophore group and lacking phytol. According to this interpretation the compound would then be more closely related to protochlorophyll than to chlorophyll. Further work is contemplated when more material becomes available.

[1] Strain, H. H., and Manning, W. M., *J. Biol. Chem.*, **144**, 625 (1942). Strain, H. H., Manning, W. M., and Hardin, G., *J. Biol. Chem.*, **148**, 655 (1943).

[2] Ludwig, E. E., and Johnson, C. R., *Ind. and Eng. Chem., Anal. Ed.*, **14**, 895 (1942).

[Reprinted from The Journal of Experimental Medicine, April 1, 1949,
Vol. 89, No. 4, pp. 425–430]

ELECTRON MICROSCOPE STUDIES OF CELLS BY THE METHOD OF REPLICAS*

By ALBERT CLAUDE, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

Plates 16 and 17

(Received for publication, December 15, 1948)

The technique of electron microscopy has failed thus far to find general application in the study of cells and tissues, chiefly because of the difficulties involved in the preparation of specimens of required thinness. Formed elements can be isolated from the cells and examined separately (2, 3) but in this case the connections with the other cell structures are lost and a study of the general cell organization is not possible. The use of tissue culture has offered an opportunity to examine certain portions of the cells, especially thinly extended cells margins (4), but in this case also a number of limitations must be recognized: the center of the cell, as a rule, remains too thick for study; cells in tissue culture, especially actively growing ones, have a tendency to dedifferentiate; and finally, the cellular arrangement existing in the whole organ is not retained by the outgrowing cells. It would seem, therefore, that the method of sectioning, so widely successful as an adjunct to light microscopy, would be the technique of choice because of its applicability to all cells and tissues, irrespective of their individual constitution or origin. The early attempts to adapt the method of sectioning to electron microscopy have not been completely satisfactory (5–7), and experience of recent years indicates that the problem does not reside solely in the difficulties in producing sections of sufficient thinness, but also in the fact that the current methods of fixation and embedding fail to preserve the fine structure of the cells (8–10).

As is known, the absorption and scattering of electrons are not affected by molecular differences, as is the case with light of various wave lengths, but are determined by variations in atomic densities. In biological material, therefore, where the major elements, *i.e.* carbon, nitrogen, and oxygen have nearly the same atomic weights, the absorption and scattering observed in the electron microscope is a measure, but in inverse proportion, of the relative amount of water originally present in different parts of the fresh specimen. The experiments reported in the present paper take advantage of this fact, and are based on the assumption that differences in the distribution and concentration of substance, as occurring in cells and tissues find expression, after

* The results recorded in this paper were presented at the annual meeting of the Electron Microscope Society of America, December 11 to 13, 1947, in Philadelphia (1).

drying, in variations in height and shape at the surface of the specimens. The results here reported indicate that surface replicas of dried cells and tissues can depict with surprising accuracy morphological details of their internal constitution.

Material and Methods

In the present work the material studied has been single cells, as in blood smears or tissue imprints, and bacterial cells. The preparations were fixed, usually in osmium tetroxide vapors, and allowed to dry in air, or over P_2O_5.

The technique employed consisted in preparing replicas of the cellular material and recording in the electron microscope the negative image so obtained, a method heretofore used in the study of crystalline structures or metal surfaces (11, 12).

Replicas were obtained by immersing glass slides supporting the dried specimen in a 0.5 per cent solution of formvar E[1] in ethylene dichloride and allowing the preparation to dry while in the horizontal position. The resulting plastic film presented a level surface on its upper side, while retaining the imprint on its under surface of the irregularities reflected at the surface of the specimen. The difficulties encountered in detaching the mold, prior to its transfer to the supporting wire mesh of the electron microscope, varied greatly with the nature, and especially the thickness, of the specimen. The plastic film must be thin to insure contrast in the image made of the replica and its fragility precludes the use of much mechanical force when freeing it from the glass support; hence, conditions must be such that it can be detached readily. This is usually accomplished when the cellular elements projecting into the film, and therefore weakening it in places, are not crowded, but have between them a sustaining network of relatively thick film. Favorable conditions of this sort are provided by using thinly spread blood or bacterial smears, where some free space is left between the cells.

The preparation of the mount, *i.e.* the lifting of the replica from the cells, and its transfer to the wire mesh screen of the electron microscope was carried out under the dissecting microscope. Formvar-coated smears of blood or bacteria were immersed in distilled water and an area, about the size and the shape of the supporting screen, was selected and outlined. This film disc was detached by means of sharpened watchmaker's forceps, and moved over a screen placed alongside beforehand. The screen and the film over it were then lifted from the water and the preparation was drained on blotting paper and allowed to dry. When resorting to shadowing of the replicas, the film was detached, turned over, and spread on a clean glass slide, in the inverted position. The exposed side of the replica was then shadowed in the usual manner.

The observations were made by means of a RCA, type E.M.U., electron microscope.

EXPERIMENTAL

Replicas of Blood Cells.—Fig. 1 shows a micrograph of a replica of a smear of chicken blood. The replica was mounted on the screen of the electron microscope, and the photograph was made with an ordinary microscope, using visible light. The smears had been prepared in the usual manner by spreading thinly on a glass slide a drop of blood obtained from the comb of an apparently normal pullet, fixing it rapidly over osmium tetroxide vapors, and allowing it to dry in air. As shown in Fig. 1, the replica of the smear produces an image

[1] Formvar E (grade No. 15–95), obtained from the Shawinigan Products Corporation, New York.

of the blood cells, so faithful that it is difficult to distinguish, under the light microscope, between the original unstained cells and their plastic mold. As in the direct light microscope examination of the smear itself, the nuclei in the replicas of the erythrocytes are evident, and the leucocyte in the field appears to contain granules. Fig. 2 represents the replica of a chicken erythrocyte, photographed with the electron microscope under a magnification of 2200, and enlarged to 5300. Nearby is what appears to be the "ghost" of a red cell, with remnants of its nucleus. The fact that the body of the apparently intact erythrocyte appears granular, whereas the ghost cell is smooth and homogeneous, is evidence for difference in their surface conditions, or the properties of their membranes. The background of the preparation in Fig. 2 presents a fine granular structure, presumably produced by elements of the plasma.

Fig. 3 shows an electron micrograph of the replica of a mouse leucocyte, apparently a monocyte or a large lymphocyte. About it, can be seen the curved margins of a number of red cells. The replica reveals internal details, notably the shape of the nucleus, and cytoplasmic bodies with the appearance of rod-shaped mitochondria.

Fig. 4 is a micrograph of a chicken platelet, showing the vague outline of what is probably a nucleus, three vacuoles, and small bodies of various shapes which may be mitochondria. Fig. 5 is the replica of a leucocyte of chicken blood. The numerous dark bodies shown in the electron micrograph represent depressions in the surface of the dried cell and probably correspond to what were vacuoles. Differences in the density to the electron beam in these areas may reflect differences in the amount of water which existed originally in the various vacuoles.

Replicas of Bacteria.—Microorganisms are generally surrounded by a voluminous capsule or are encased in a relatively rigid covering. *A priori*, it would seem likely that structural details of their cell content would not be accessible for study by the method of replicas. In fact, it has been possible to obtain replicas, of *E. coli* for the most part, which picture a number of morphological features reflecting, presumably, details of cellular organization of the bacteria.

Figs. 6 to 8 are micrographs of such replicas. Fig. 6 shows a cell with two large bodies, one near the center, and the other near one end. Fig. 7 shows two bacterial cells, one of them appearing but faintly, obviously because it lay partly beneath the other and made but a slight impression on the formvar film. The upper cell shows two terminal bodies, like those of growing organisms, and a central body of smaller size. Fig. 8 represents a portion of a filamentous form of *E. coli*, such as develops in aging cultures. The white bodies which appear in the organism seem to be arranged along a spiral path extending from one to the other end of the filament. The same bodies have been noted in replicas of similar elongated organisms. They appear to be sufficiently

rigid to cause the cell wall to rupture over them, probably during desiccation; in the picture this is noticeable at both ends of the filament. The nature of inclusions of identical morphology detected with the ordinary microscope in specimens of various forms of *E. coli* is not known; the fact that they stain with methyl green might be taken to indicate that they contain in appreciable amounts substances related to chromatin and that they represent bacterial nuclei.

DISCUSSION

Replicas of the surface of certain cells have occasionally been obtained and photographed in the electron microscope (13, 14) but it seems that full advantage has not been taken of this interesting technique, and the fact that it can furnish information concerning the internal structure of cells has not been realized. These have been the objects of the work reported in the present paper. Replicas of blood cells and bacteria have been obtained which not only give the shape of the cells but show nuclear membranes and what appears to be chromatin structures, mitochondria, and vacuoles. An important feature of the method is that the thickness of the specimen, often a limiting factor in the electron microscopy of cells, may no longer be significant if replicas of the proper thinness can be prepared. The method would appear useful in the study of erythrocytes and bacteria, and in the case of the nuclear region, which remains too thick for direct electron microscopy even in thinly extended cells in tissue cultures. That the method of replicas, as applied to the study of cells, can be technically improved is probable, so that higher resolution may be obtained and even finer details of internal structure may be revealed. The hope seems warranted that the method may assist in the study of problems to which direct electron microscopy cannot yet be applied, as in the case of the intracellular growth of malarial parasites, the penetration and growth of bacterial viruses, and the morphology of chromosomes.

SUMMARY

The method of replicas has been applied to the study with the electron microscope of blood cells and bacteria.

The results indicate that the method can reveal details of intracellular structures. Nuclei can be perceived, and also cytoplasmic bodies such as mitochondria and vacuoles.

BIBLIOGRAPHY

1. Claude, A., *J. Appl. Physics*, 1948, **19**, 126.
2. Schmitt, F. O., *Harvey Lectures*, 1944–45, **40**, 249.
3. Claude, A., and Fullam, E. F., *J. Exp. Med.*, 1945, **81**, 51.
4. Porter, K. R., Claude, A., and Fullam, E. F., *J. Exp. Med.*, 1945, **81**, 233.

5. von Ardenne, M., *Z. wissensch. Mikr.*, 1939–40, **56,** 8; 1940, **57,** 291.
6. Richards, A. G., Jr., Anderson, T. F., and Hance, R. T., *Proc. Soc. Exp. Biol. and Med.*, 1942, **51,** 148.
7. Sjöstrand, F., *Nature*, 1943, **151,** 725.
8. Claude, A., and Fullam, E. F., *J. Exp. Med.*, 1946, **83,** 499.
9. Claude, A., *Harvey Lectures*, 1947–48, **43,** in press.
10. Pease, D. C., and Baker, R. F., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67,** 470.
11. Zworykin, V. K., and Ramberg, E. G., *J. Appl. Physics*, 1941, **12,** 692.
12. Schaefer, V. J., and Harker, D., *J. Appl. Physics*, 1942, **13,** 427.
13. Hillier, J., and Baker, R. F., *J. Bact.*, 1946, **52,** 411.
14. Groupé, V., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64,** 401.

EXPLANATION OF PLATES

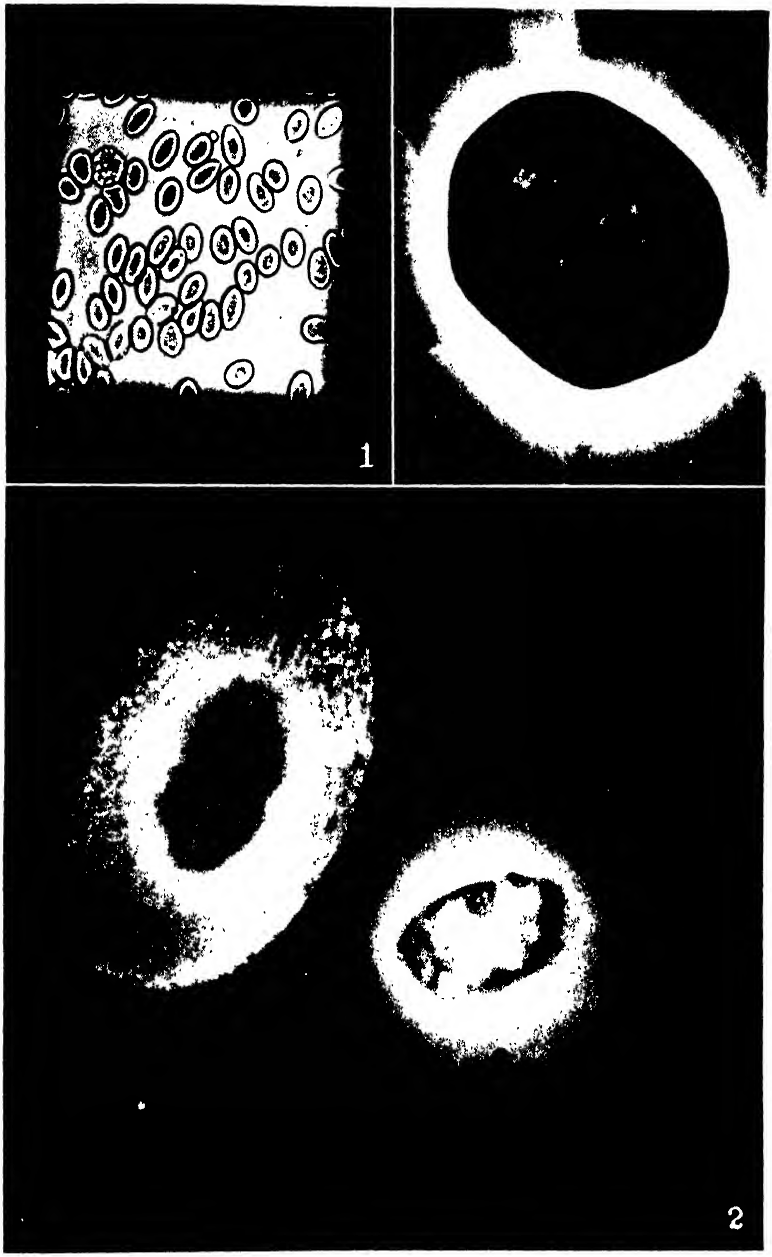
All preparations illustrated in Figs. 1 to 8 were fixed by exposure to osmium tetroxide vapors for 5 to 15 minutes, and subsequently were allowed to dry in air. Since the pictures were made from replicas, all illustrations are negative images of the original cells.

PLATE 16

FIG. 1. Formvar replica of a smear of chicken blood. The imprints left on the plastic film reproduce to a striking extent the appearance of the original blood cells as viewed without staining. The picture shows many erythrocytes, and one leucocyte. Photograph made with an ordinary microscope and visible light at a magnification of 250, enlarged to 450.

FIG. 2. Formvar replica of chicken blood cells. On the left, is an apparently intact erythrocyte; on the right, what would appear to be the ghost of a red cell. Electron micrograph taken at a magnification of 2200, and enlarged to 5300.

FIG. 3. Replica of mouse leucocyte, probably a monocyte, or a large lymphocyte. The elongated bodies in the cytoplasm correspond to mitochondria. Electron micrograph taken at a magnification of 2600, and enlarged to 4000.



(Claude: Electron microscope studies of cells)

PLATE 17

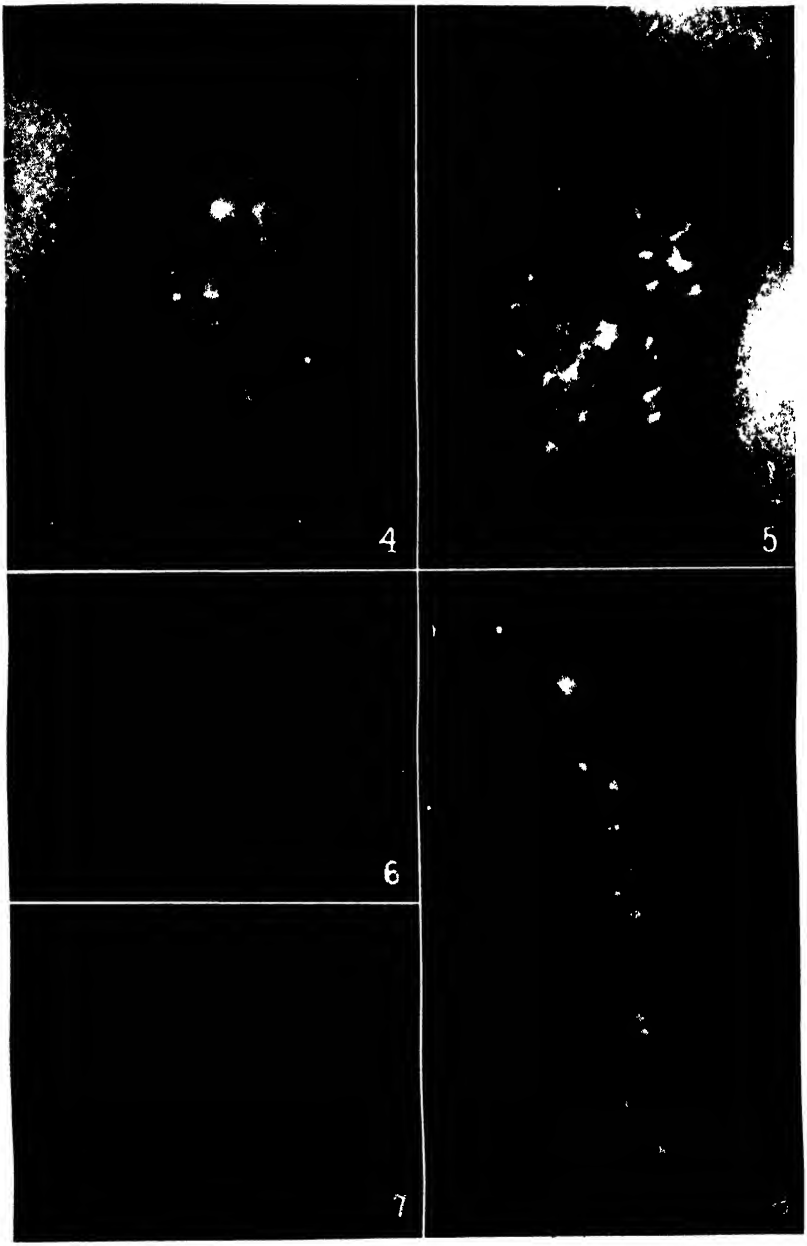
FIG. 4. Replica of a chicken platelet. A nucleus is faintly outlined. The dark cytoplasmic bodies presumably correspond to vacuoles, the light bodies probably to mitochondria, judging from the bulges and depressions, respectively, that they left on the replica. Electron micrograph taken at a magnification of 2600, and enlarged to 5200.

FIG. 5. Replica of a chicken leucocyte. The areas free of granules correspond to two lobes of the polymorphic nucleus. Electron micrograph taken at a magnification of 2200, and enlarged to 4400.

FIG. 6. Replica of an organism from an *E. coli* culture, with two relatively large internal bodies, one near the middle of the cell, the other near one end. Electron micrograph taken at a magnification of 2600, and enlarged to 7800.

FIG. 7. Replica of organisms from an *E. coli* culture, showing one cell lying partly over another. The upper cell presumably made the more effective replica. It has one rounded body at each end, and a central one of smaller size. Electron micrograph taken at a magnification of 2600, and enlarged to 7800.

FIG. 8. Replica of a filamentous organism, of a type frequently found in "old" cultures of *E. coli*. The picture shows discrete bodies, apparently arranged along a spiral path. In ordinary microscopic preparations, methyl green stains bodies similar to those shown, and also to the larger ones of Figs. 6 and 7. Electron micrograph taken at a magnification of 2600, and enlarged to 5200.



(Claude: Electron microscope studies of cells)

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, April 1, 1949, Vol. 89, No. 4, pp. 431–438]

AN ELECTRON MICROSCOPE STUDY OF SALIVARY GLAND CHROMOSOMES BY THE REPLICA METHOD

BY SANFORD L. PALAY, M.D.,* AND ALBERT CLAUDE, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

PLATES 18 TO 20

(Received for publication, December 15, 1948)

Information concerning the structure of the giant chromosomes of dipteran salivary glands has derived mainly from the application of cytological procedures, histochemical techniques, and micromanipulation (1), methods which have utilized the limited resolving power of the light microscope. No studies have been recorded in which the increased resolution afforded by the electron microscope has been used to elucidate the organization of the giant chromosomes, although a number of attempts have been made to study the chromosomes of plants (2, 3), mammals (4), and birds (5) by means of this instrument. Such attempts have been impeded by the thickness of the chromosomes and their opacity to the electron beam; hence little or no fine structure has been revealed. In the present investigation this technical difficulty was obviated by resorting to a modification of the simple replica method (6) used previously in the study of metal surfaces. Replicas of salivary gland chromosomes obtained by this method reveal considerable detail not apparent in electron micrographs of the original chromosomes.

Material and Method

Preparation of Chromosomes.—Full grown larvae of *Drosophila pseudoobscura* and *Drosophila melanogaster* Sc^8w^a and Ore R, cultured on corn meal–molasses–agar, were used for this study.[1] The larvae were placed in a drop of 45 per cent acetic acid and the salivary glands were isolated under the dissecting microscope by means of stainless steel needles. Adherent fat body was discarded. Only those glands filled with secretion and composed of large, flat cells were selected for study. The large-cell tip of the gland was then transferred to a fresh drop of 45 per cent acetic acid on a glass slide usually coated beforehand with a thin film of Mayer's albumen. A coverslip was laid over the preparation, the slide was inverted onto a paper towel, and the cells were crushed by the application of pressure through the slide. By this maneuver the tissue was spread out very thin and flat so that Liesegang's rings appeared. Examination under the light microscope showed that in successful preparations practically all the cells had been disrupted, with the chromosomes dispersed, individually extended, or stretched out, many of them isolated and apparently free from nuclear and cytoplasmic debris. For further fixation and progressive dehydration these preparations were exposed to

* Fellow in the Medical Sciences of the National Research Council.

[1] The original cultures of these larvae were kindly provided by Mr. Bruce Wallace of the Carnegie Institution of Washington, Cold Spring Harbor, New York, for whose continued interest and aid we are grateful.

alcohol vapors for 12 to 24 hours by keeping them in a closed Coplin jar containing just enough 95 per cent alcohol to reach the lower edges of the coverslips. The preparations were finally immersed in 95 per cent ethyl alcohol for at least 24 hours.

Preparation of Replicas.—The coverslip was carefully pried loose from the slide while still immersed in 95 per cent alcohol. The slide and its separated coverslip were each passed through three changes of fresh absolute ethyl alcohol and placed in a desiccator over phosphorus pentoxide until all visible moisture had vanished.

In many preparations the chromosomes remained adherent to the coverslip rather than to the slide after they had been separated. When the coverslip was used in making replicas it was first cemented with clarite to a glass slide, tissue side up.

Replicas were made by dipping the slide vertically into a 0.75 per cent solution of formvar[2] in ethylene dichloride just long enough to immerse and withdraw. Upon removal the slide was kept in the vertical position while the excess formvar was drained off, the back of the slide was wiped with a gauze pad, and the film was allowed to dry in air. When the humidity was high it was necessary to dry the film in a phosphorus pentoxide desiccator, because the cooling effect of the rapidly evaporating ethylene dichloride would cause water to condense on the film, which would then become opaque and brittle.

Stripping of the film from the slide was carried out in water under the dissecting microscope by means of sharpened watchmaker's forceps. In regions where the chromosomes appeared properly spread out and numerous, circular areas were outlined and discs of the film, just large enough to cover the specimen screen of the electron microscope, were peeled off and brought to the surface of the water where they immediately flattened out, and picked up on 160 mesh stainless steel screens. The screens with their films were drained by touching them to a lintless towel and were allowed to dry in air. Replicas thus obtained were then ready for examination in the electron microscope. The whole procedure of coating the slide with formvar and mounting the film on the specimen screen took about 15 minutes.

Chromosome preparations from which replicas have been obtained can be used repeatedly for the same purpose if the slide is freed of remaining formvar by stripping and then dried after passage through several changes of absolute alcohol.

All micrographs were made with the RCA (type E.M.U.) electron microscope.[3]

Comments on the Method.—Dehydration and complete drying of the spreads were found necessary for successful preparation of replicas. If the material dried from an aqueous medium, the chromosomes shrank considerably and were distorted. If the spread was not allowed to dry in the air, but was passed from absolute alcohol through ethylene dichloride into the formvar solution, the chromosomes became embedded in the film and came off the slide with it. Even when the slide had been dried in air, the chromosomes would come off with the film if the slide was allowed to stay in the formvar solution too long. It is necessary to withdraw the slide as soon as it has been immersed.

The thickness of the chromosomes is another important factor in making replicas. Good replicas could be made only when the dried chromosomes were sufficiently thin, as indicated by their transparency and lack of distortion when examined with the high dry power of the light microscope (× 600). Such chromosomes were almost invisible unless examined by diffused or diminished light. The thickness of these preparations depended upon the pressure applied in crushing the salivary glands and the length of time they were held between slide and coverslip in the alcohol vapor. If the preparation was too thick the chromosomes shrank

[2] Formvar E (grade No. 15–95), obtained from the Shawinigan Products Corporation, New York.

[3] Made available through the courtesy of Dr. R. M. Taylor, Director of the laboratories of the International Health Division of The Rockefeller Foundation.

in drying and appeared opaque and distorted. Thus the appearance of the dried chromosomes under the light microscope indicated whether they were suitable for the preparation of replicas.

A certain thickness of the formvar film was also found to be essential for the success of the method. Films that were too thin were not only difficult to strip but also gave no detail. Films that were too thick could be detached readily but gave poor contrast in the electron microscope and exhibited many defects, such as tears and holes in the replicas. Whether the film was of optimal thickness could be readily ascertained while it was still on the slide immersed in water. Under favorable conditions numerous tiny water droplets penetrate under the film, causing minute elevations which have a golden color when viewed under the dissecting microscope. Such a film can be easily detached from the slide and when placed on a screen and dried has a golden color in daylight (angle of incidence approximately 80°). According to Schaefer and Harker (6), this color is given by a film 70 mμ in thickness, but measurements of our films with polarized light[4] revealed an average thickness in the area of the replicas of about 130 mμ.

Care had to be taken that the slides and coverslips were free from all traces of grease, for this prevents stripping of the plastic film. Only new slides and coverslips were used, cleaned in sulfuric acid–bichromate solution and dried from alcohol.

Numerous attempts were made to change the fixation of the chromosomes in order to improve the definition of the replicas. Osmium tetroxide, trichloracetic acid, phosphotungstic acid, and Flemming's mixture were all tried in various combinations, but without success. Either the chromosomes were not preserved or replicas could not be made from them.

Experimental.—In an attempt to localize desoxyribonucleic acid in the chromosomes, dried squash preparations of salivary glands were subjected to digestion by desoxyribonuclease (7) in a mixture containing, as final concentrations, 0.01 mg. enzyme[5] per ml., 0.025 M phosphate buffer of pH 7.3, and 0.005 M magnesium sulfate. Control mixtures were identical except that no desoxyribonuclease was added.

The salt mixtures were prepared on the day before the experiment and were kept at 37°C. overnight. The dry enzyme was dissolved in the warm salt mixture just before use. Both the digesting and control mixtures had a final pH of 7.45. The preparations were incubated at 37° for 60 minutes, washed in five changes of distilled water, passed through three changes of fresh absolute alcohol, and dried in air for 3 days. Formvar replicas of the chromosomes were then made in the usual manner. After the replicas had been taken, both controls and the digested preparations were treated according to the Feulgen nucleal technique.

OBSERVATIONS

As the replicas described in this report are, in effect, casts of the original chromosomes, the electron micrographs obtained from them are negative images. Therefore, in the final prints the thinner portions of the chromosomes are represented by dark grey or black, and the thicker portions by light grey or white.

Because the replicas were surface impressions it was essential that the chromosomes be separated from one another and be free of overlying debris which would obscure structural details. For this reason the replicas studied

[4] We are indebted to Dr. A. Rothen of The Rockefeller Institute for Medical Research, who made the measurements.

[5] The sample of partially purified desoxyribonuclease was kindly supplied by Dr. M. McCarty of the Hospital of The Rockefeller Institute for Medical Research

were selected from those preparations in which the chromosomes appeared untangled and cleanly separated from cellular debris. Even so, it was not possible to obtain a complete, satisfactory record of a whole chromosome, because twists and turns sometimes distorted considerable portions of it and because the thicker bands often revealed little or no detail. Since moderately stretched chromosomes of *Drosophila*, exclusive of the short fourth chromosome, are 220 to 485 μ in length (8), an entirely uncoiled chromosome would extend across several squares of the specimen screen, and appreciable portions would be hidden by the intervening wires of the screen. Hence a complete picture of an uncoiled chromosome could not be made.

As can be seen from an examination of Figs. 1, 2, and 3, the giant salivary gland chromosome in the unstretched condition appears to consist of a series of closely apposed rows or bands of small round bodies extending across its width. In the clearest band in Fig. 1, a row of bodies can be discerned measuring from 250 to 330 mμ in diameter. Between granules and between rows there are no obvious connections. The discreteness of the bodies can be seen in Fig. 2 where the chromosome is slightly stretched. In Fig. 3 the striated and granular structure is also evident, with no indication of strands or threads between granules or bands. In all the micrographs these granules have been measured wherever their contours were clearly discernible. They were found to vary from 210 to 330 mμ in diameter, the majority being in the range of 250 to 290 mμ. Since these granules are therefore just at the limit of resolution of the light microscope, they are considered to be identical with the small basophilic granules and vesicles that have been designated as chromomeres (1).

The micrographs of the replicas reveal no evidence of a limiting membrane surrounding the chromosomes, either in lax or stretched specimens. The chromomeres extend to the edge of the chromosome, and no sheath or pellicle appears to intervene between the outermost chromomeres in a band and the material around the chromosome (Figs. 1, 3, and 4). A definite matrix of intrachromosomal substance between chromomeres also appears to be absent.

When chromosomes are moderately or greatly stretched, the bands of chromomeres separate, and more or less coarse longitudinal strands appear between them (Figs. 4, 5, and 6). In some places these filaments stretch between corresponding granules of neighboring bands as at *a* in Fig. 4; in other places (*b* in Figs. 4 and 5) they form an interweaving longitudinal meshwork. Nowhere could the same strand be clearly traced across more than one row of granules. The replica method has not revealed any fine periodic structure in the strands such as has been found in myofibrils or collagen threads (9). They seem, on the contrary, to be homogeneous fibers which divide and anastomose between bands, their configuration depending upon the amount and direction of the stresses developed in stretching the chromosomes (Fig. 6).

The digestion experiments with desoxyribonuclease provided further infor-

mation about the strands. Figs. 7 and 8 show portions of moderately and greatly stretched chromosomes after they had been subjected to the action of desoxyribonuclease for 60 minutes at 37°C. These chromosomes give a negative Feulgen reaction when tested after the replicas had been made, thus indicating that the desoxyribonucleic acid had been removed by the digestion. The continuity of the chromosomes was not affected by the procedure but the chromomeres appeared to be more discrete than those in untreated preparations. In the heavier bands small granules can be discerned that are not obvious in such bands of undigested chromosomes (compare Fig. 7 with Fig. 3). The distinct chromomeres, measuring from 210 to 250 mμ in diameter, were also a little smaller than those of the control chromosomes. Between the bands no fibers or filaments remained, but in their place was an amorphous material that had no apparent orientation in relation to the chromomeres. The control preparations resembled in all respects the untreated chromosomes (compare Fig. 8 with Figs. 4 and 5).

DISCUSSION

The replica method has already been applied to blood cells and bacteria with some success (10), and it seems probable that with certain modifications it can be used to study tissues which have thus far proved inaccessible to electron microscopy because of their opacity to the electron beam. In the present study on giant salivary gland chromosomes by means of the replica technique, no detail has been revealed that had not been suggested by examination of stained preparations with the light microscope. However, the increased resolution and magnification provided by the electron microscope have made it possible to characterize the structure of these chromosomes more fully.

According to the most widely accepted view, the giant chromosomes consist of a number of parallel and closely approximated threads or chromonemata bearing homologous chromomeres at regular intervals marked by Feulgen-positive, basophilic bands. This polytene structure was postulated by Koltzoff (11) and Bridges (8) on the basis of fixed and stained preparations and has been supported by the work of Bauer (12), Painter and Griffen (13), and d'Angelo (14). Metz and Lawrence (15) have presented contrary evidence to the effect that the chromosomes are alveolar in structure, made up of achromatic vesicles or droplets in a chromatic matrix, and that the strands visible in stretched chromosomes are not true chromonemata but artifacts, stress lines in the matrix produced by the stretching of chromatic material from the band regions. Buck's micromanipulation studies (16) on fixed salivary gland chromosomes have tended to confirm this interpretation. The polytene theory has also been challenged by Ris and Crouse (17), who state that the bands are actually caused by the complex coiling of a bundle of chromonemata which weave back

and forth across the width of the chromosome. According to this view the chromonema is uniformly Feulgen-positive and the so called chromomeres are optical sections of gyres in the chromonema. The electron micrographs presented in this paper do not substantiate any one of these theories.

Although there is general agreement (1) that a sheath envelops the chromosomes, we could find no evidence for it in the replicas. Chromomeres occupy the entire width of the chromosomes. It is possible that the sheath may be too delicate to withstand squashing of the chromosomes, but the fact that the nuclear membranes of blood cells can be demonstrated in replicas (10) makes it seem improbable that the replica technique would not disclose a sheath in the chromosomes if it were present. However, in view of the apparently conclusive micrurgical demonstration by d'Angelo (14) of the existence of a membrane in the living giant chromosomes of *Chironomus*, it may be inferred that in our material the sheath was destroyed or dispersed by the process of making squash preparations.

The existence and character of a chromosomal matrix in which the chromonemata and chromomeres are embedded have been controversial subjects for many years (1). The observations reported here provide no evidence for the presence of any intrachromosomal substance distinct from the chromomeres. Neighboring rows of chromomeres lie in very close apposition in the unstretched chromosomes. The appearance of achromatic interband regions in stained lax chromosomes may be merely the result of the relatively poor nucleic acid content of certain bands.

The nature of the strands seen in electron micrographs of the interband regions of stretched chromosomes is more difficult to determine. The facts that these filaments are not apparent in the lax chromosomes, that they are evident only in stretched preparations, that they divide and anastomose between bands, and that they disappear when digested by desoxyribonuclease suggest that they are artifacts, as Metz and his coworkers maintain. Some indication of their nature can be obtained by comparing Fig. 5 in the present paper with the electron micrographs of thymonucleohistone in the paper of Mazia, Hayashi, and Yudowitch (18). These workers compressed films of thymonucleoprotein into fibers which under the electron microscope appear as narrow, thin, anastomosing sheets, resembling the strands that form between bands of stretched chromosomes. This similarity is further borne out by the results of our digestion experiments with desoxyribonuclease. These results suggest that when the chromosome is stretched desoxyribonucleoprotein is pulled out from the surface of adjoining chromomeres into sheets extending between them and that these sheets tear or shred into strands according to the irregular stresses produced by pressure on the coverslip and the varying resistances of the bands. When nucleic acid is removed by enzyme action, the strand-like appearance is destroyed and only the protein remains as amor-

phous material. This interpretation of the strands, furthermore, is in accord with the fact that the interband regions of salivary gland chromosomes are positively birefringent to polarized light only when in the stretched condition (19).

The electron micrographs show that the transverse bands are indeed composed of small round granules arranged in rows across the width of the chromosomes. Since the replicas are surface impressions there can be no possibility of confusion arising from misinterpretation of optical sections. It is difficult to visualize how the small round bodies seen in these preparations could be caused by gyres of a continuous uniform thread. Moreover, the persistence of these bodies after the chromosome has been subjected to digestion by desoxyribonuclease indicates that they have a complex constitution. They contain at least two types of substances, desoxyribonucleic acid and other constituents, probably protein in nature. Since the granules are smaller after digestion and since strands that are destroyed by desoxyribonuclease appear between them when the chromosome is stretched, at least part of the desoxyribonucleic acid lies on the surface. The fact that after digestion granules are more readily discerned in the heavy bands also supports this inference. Evidently the nucleic acid component does not determine the shape of the chromomeres.

In general the observations reported here lead to the concept that the giant salivary gland chromosome is essentially chromomeric in structure. The chromomeres lie in close apposition to one another to form continuous, parallel, adherent chains without interpolated threads. Each member of a chromosome pair is, then, a single giant chromonema composed of chromomeres that are more numerous, or perhaps larger, or both, than those in the chromosomes of ordinary cells.

SUMMARY

A method for preparing replicas of salivary gland chromosomes for electron microscopy is described.

Electron micrographs of these replicas show that the giant chromosomes are composed of a series of small granules of approximately equal size arranged transversely across the chromosome.

In stretched preparations a linear network of filaments appears between the rows of granules. These fibers cannot be traced between corresponding granules of more than two consecutive rows. When the chromosomes are digested by desoxyribonuclease, these fibers disappear and only amorphous material remains between the bands. The characteristics of the strands suggest that they are artifacts produced when the chromosomes are stretched.

The small granules are composed of desoxyribonucleic acid and at least one other component, probably a protein. The nucleic acid seems to lie at least in part on the surface of each granule.

BIBLIOGRAPHY

1. Kautmann, B. P., *Bot. Rev.*, 1948, **14**, 57.
2. Elvers, I., *Ark. Bot.*, 1942, **30B**, No. 4, 1.
3. Buchholz, J. T., *Am. J. Bot.*, 1947, **34**, 445.
4. von Ardenne, M., *Z. Physik*, 1940, **115**, 339.
5. Hovanitz, W., *Genetics*, 1947, **32**, 500.
6. Schaefer, V. J., and Harker, D., *J. Appl. Physics*, 1942, **13**, 427.
7. McCarty, M., *J. Gen. Physiol.*, 1946, **29**, 123.
8. Bridges, C. B., *J. Hered.*, 1935, **26**, 60.
9. Schmitt, F. O., *Harvey Lectures*, 1944–45, **40**, 249.
10. Claude, A., *J. Exp. Med.*, 1949, **89**, 425.
11. Koltzoff, N., *Science*, 1934, **80**, 312.
12. Bauer, H., *Z. Zellforsch. u. mikr. Anat.*, 1935, **23**, 280.
13. Painter, T. S., and Griffen, A. B., *Genetics*, 1937, **22**, 612.
14. d'Angelo, E. G., *Biol. Bull.*, 1946, **90**, 71.
15. Metz, C. W., and Lawrence, E. G., *Quart. Rev. Biol.*, 1937, **12**, 135.
16. Buck, J. B., *J. Hered.*, 1942, **33**, 2.
17. Ris, H., and Crouse, H., *Proc. Nat. Acad. Sc.*, 1945, **31**, 321.
18. Mazia, D., Hayashi, T., and Yudowitch, K., in Cold Spring Harbor Symposia on Quantitative Biology, Cold Spring Harbor, Long Island Biological Association, 1947, **12**, 122.
19. Pfeiffer, H. H., *Chromosoma*, 1941, **2**, 77.

EXPLANATION OF PLATES

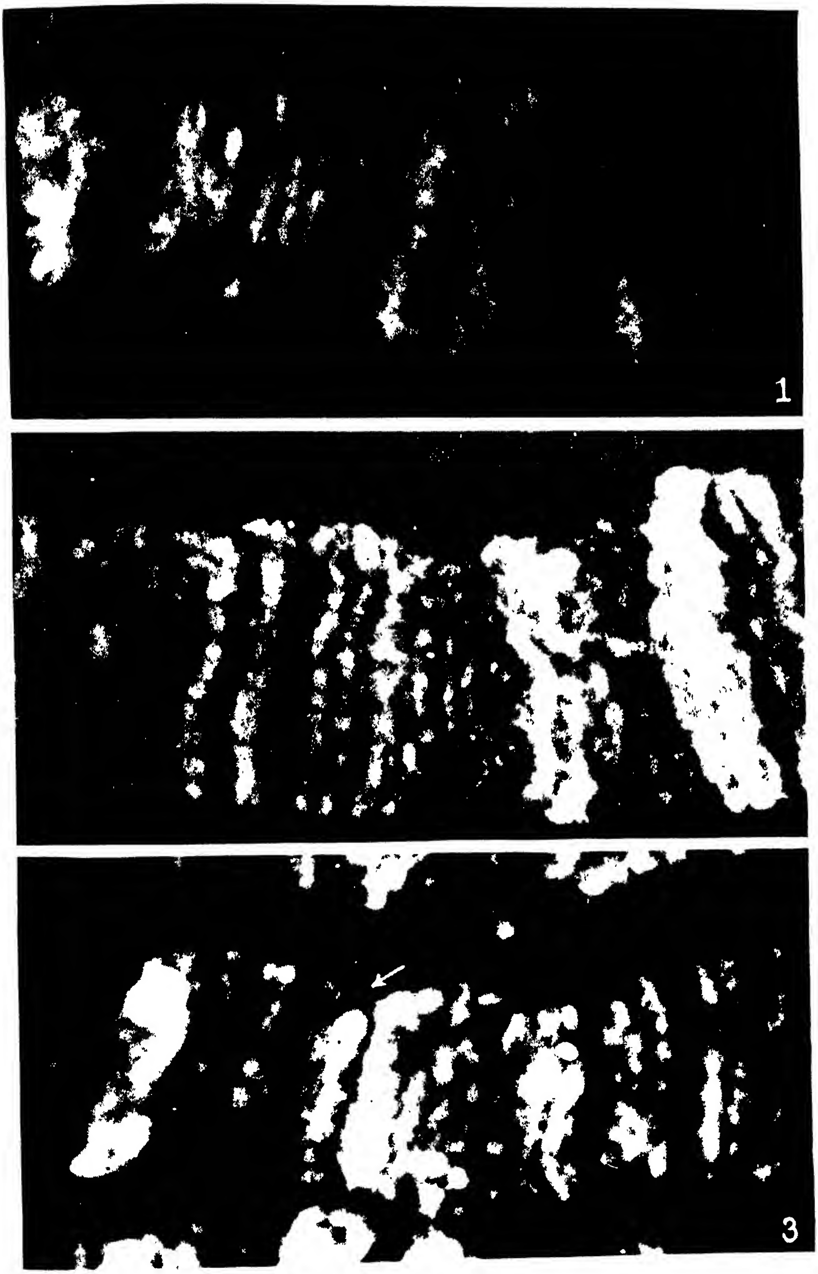
All figures are electron micrographs of formvar replicas made from giant salivary gland chromosomes fixed in 45 per cent acetic acid and dehydrated and dried from absolute ethyl alcohol. The thicker portions of the chromosomes are represented by light grey or white, the thinner portions by dark grey or black.

PLATE 18

FIG. 1. Portion of an unstretched chromosome of *D. melanogaster* Sc^{8}w^{a}. The rows of granules (chromomeres) are close together, with no apparent interconnecting threads. Micrograph taken at a magnification of 2200, enlarged to 8140.

FIG. 2. Part of a slightly stretched chromosome of *D. melanogaster* Sc^{8}w^{a}, showing rows of chromomeres separated by short distances. Fiber formation is restricted to the lower edge of the chromosome. The thin, diagonal, dark line (arrow) extending along the length of the chromosome marks the groove between the two component chromatids coiled loosely about each other. Micrograph taken at a magnification of 2200 enlarged to 8140.

FIG. 3. Portion of a chromosome of *D. melanogaster* Sc^{8}w^{a}, showing chromomeric structure and coiling of the chromatids. The arrow points to the groove between chromatids. Micrograph taken ta a magnification of 2600, enlarged to 9620.



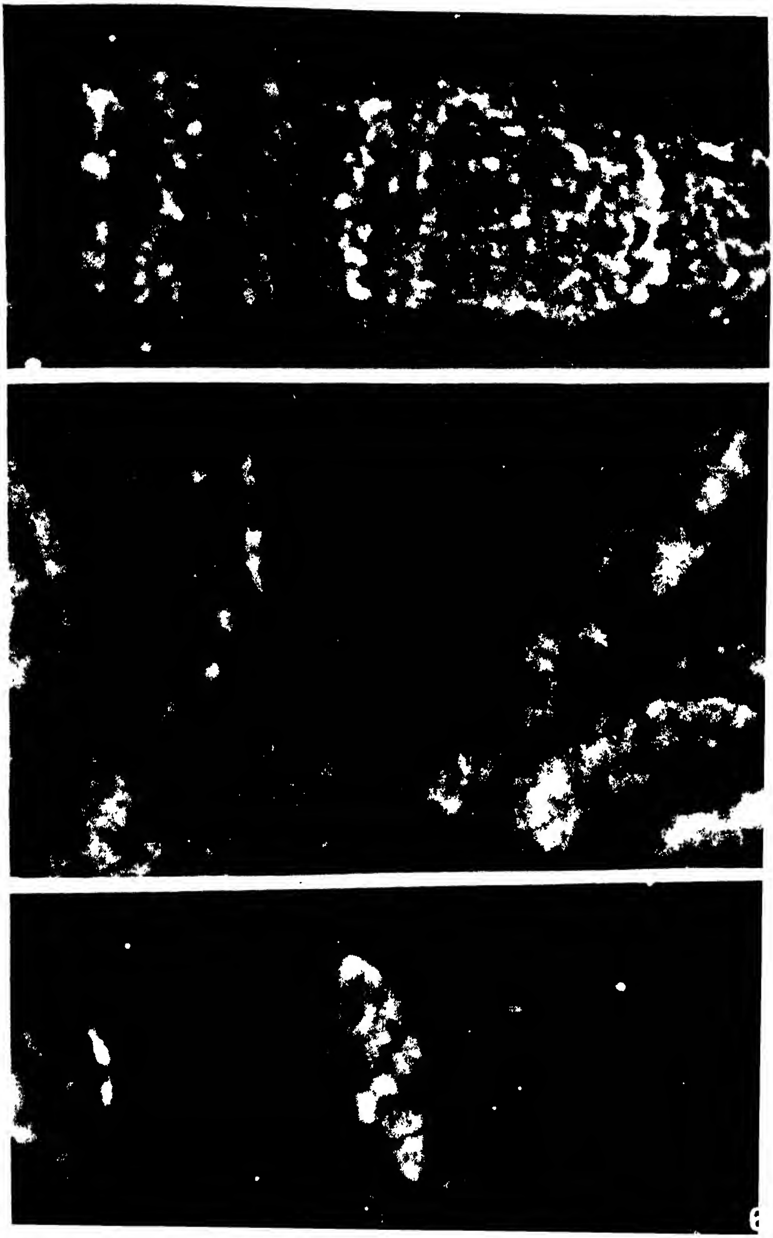
(Palay and Claude: Electron microscopy of salivary gland chromosomes)

PLATE 19

FIG. 4. Portion of a stretched chromosome of *D. melanogaster* Sc^8w^a, showing at *a* the filaments extending between corresponding chromomeres of neighboring rows. At *b* the filaments are interconnected to form a meshwork. Stretching of the chromosome has somewhat separated the round bodies or granules from one another. Micrograph taken at a magnification of 2200, enlarged to 8140.

FIG. 5. Portion of a greatly stretched chromosome of *D. melanogaster* Ore R, showing meshwork of filaments between bands of chromomeres (*b*). Micrograph taken at a magnification of 2600, enlarged to 9620.

FIG. 6. Portion of a greatly stretched chromosome of *D. melanogaster* Sc^8w^a, showing two bands composed of granules approximately 240 mμ in diameter, connected by longitudinal strands. Micrograph taken at a magnification of 3100, enlarged to 9455.



(Palay and Claude: Electron microscopy of salivary gland chromosomes)

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, May 1, 1949, Vol. 89, No. 5, pp. 479-489]

THE EFFECT OF LITHIUM PERIODATE ON CRYSTALLINE BOVINE SERUM ALBUMIN

BY WALTHER F. GOEBEL, PH.D., AND GERTRUDE E. PERLMANN, PH.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, January 21, 1949)

In a recent report it was shown that the biological activities of two proteins, crystalline ribonuclease and the immune globulin of Type III antipneumococcus horse serum, are destroyed when the proteins are subjected to the action of dilute lithium periodate at physiological pH values (1). The nature of the chemical changes involved are ill understood, but that they are considerable is evident not only because of the loss in biological activity which occurs, but because marked changes in the absorption spectrum are observed following the oxidation. The present paper describes a continuation of the work. In order to gain further insight into the mode of action of the periodate ion on native proteins, a study has been made of its action on crystalline bovine serum albumin. It will be shown that the physical, chemical, and immunological properties of this protein are radically altered on contact with the reagent and that these changes are accompanied by an alteration or destruction of certain amino acids.

Materials and Methods

Crystalline bovine serum albumin was purchased from The Armour Laboratories, Chicago. Buffered lithium periodate was prepared by dissolving 2.28 gm. of $HIO_4 \cdot 2H_2O$ in 50 ml. of water; 10 ml. of 0.5 M H_3PO_4 was added followed by the addition of 20 ml. of carbonate-free 1 N LiOH. The solution was then diluted to 100 ml. The pH of this solution is 7.2.

The cystine and cysteine content of the native and oxidized proteins was determined by the method of Kassel and Brand (2). The acid hydrolysis of the various proteins was carried out as described by Brand (3). Twice distilled 6 N HCl was used, and the hydrolysis was performed in the presence of urea, and in an atmosphere of CO_2 at 130-35° for 8 hours. The sample was diluted to a known volume and aliquot portions were used for the determination of the cystine and cysteine. Tyrosine analyses were performed on alkaline hydrolysates of the proteins (4) by the procedure of Lugg (5). Tryptophane analyses were carried out directly on the intact unhydrolyzed protein as recommended by Sullivan (6). Standard curves for the absorption values of the colors developed were constructed, using in each instance the pure amino acid as standard. All determinations were made in a Beckman model DU spectrophotometer. The color developed in the cystine method was read at 850 mμ; in the tyrosine method 354 mμ; and in the tryptophane procedure 600 mμ. In all instances duplicate analyses were performed.

Electrophoretic Technique.—The electrophoretic experiments were carried out at 0.5° in the apparatus described by Longsworth (7). Samples of the native and oxidized proteins at concentrations of approximately 1.0 per cent were used. Prior to electrophoresis, the solutions were dialyzed against several portions of the same buffer as that used in the experiments.

The mobilities were computed from the descending patterns, using the bisecting ordinate of the refractive index gradient curve, and referred to 0°C.

Oxidation of Bovine Serum Albumin by Lithium Periodate.—4.0 gm. of crystalline bovine serum albumin was dissolved in 200 ml. of water; 100 ml. of 0.1 M lithium periodate buffer solution described above was then added. After standing at room temperature for 72 hours the excess periodate was decomposed by the addition of 3 ml. of 50 per cent glucose solution. The solution of the oxidized protein was thoroughly dialyzed in a rocking device against running distilled water until free of electrolytes. The end product was isolated by desiccation from the frozen state. 3.8 gm. of material was recovered. The substance was obtained as a beige-colored fluffy powder.

FINDINGS

Properties of Oxidized Bovine Serum Albumin.—When bovine serum albumin was oxidized with lithium periodate at pH 7.2, the solution gradually became yellow in color, and no visible precipitate was formed. The oxidized protein was soluble in neutral and alkaline solutions, but if the pH was lowered to pH 5.0, the solution became turbid, and at a pH of approximately 3.5 to 4.0 the protein was insoluble. The solubility of the oxidized protein in ammonium sulfate was markedly changed following oxidation with lithium periodate. At 10 per cent saturation some of the protein was precipitated, and at 30 per cent saturation the major part came out of solution. Attempts were made to crystallize the oxidized derivative from ammonium sulfate, but without success. A solution of the protein behaved in some respects like one of the native material,—the oxidized protein was precipitated by the salts of heavy metals, and by trichloracetic acid. Solutions of the oxidized protein foamed and gave a strong biuret test. The oxidized protein was also hydrolyzed by crystalline trypsin, and to a greater degree than was the native protein when the hydrolysis was carried out under the following conditions:—

Ten ml. of a 0.1 per cent solution of native and oxidized protein was heated for 3 minutes at 70° in a 0.1 molar phosphate buffer at pH 7.6. To each solution was added 500 micrograms of crystalline trypsin, and after 18 hours at 37° an aliquot of the protein was precipitated with an equal volume of 10 per cent trichloracetic acid. Analyses of the supernates revealed that 34 per cent of nitrogen was found in that of the heated native albumin, and 67 per cent in that of the oxidized protein. If the experiment was carried out on unheated protein solutions, the differences in soluble nitrogen were approximately of the same order, but the degree of proteolysis was one-third less.

Change in Absorption Spectrum of Bovine Serum Albumin Following Oxidation with $LiIO_4$.—0.4 gm. of crystalline bovine serum albumin was dissolved in 20 ml. of water; 4 ml. was removed and saved. The remainder was treated with 8.0 ml. of 0.1 M $LiIO_4$-phosphate mixture and allowed to stand at 22°. At the end of 24, 48, and 72 hours, 6 ml. samples were removed and the periodate decomposed by the addition of 0.5 ml. of 50 per cent glucose solution. All the solutions were placed in cellophane bags and dialyzed against 0.1 M phosphate buffer at pH 7.6 until free of I^- and IO_3^-. Each solution, including the control, was then diluted with the phosphate buffer until the concentration was 1.0 mg. per ml. The ultraviolet absorption spectra were determined and recorded (Fig. 1).

It is apparent from the results presented in Fig. 1 that profound changes have occurred in the ultraviolet absorption spectra of the protein after contact with the reagent. These changes become progressively greater the longer the protein is subjected to the action of the periodate ion. After 24 hour contact,

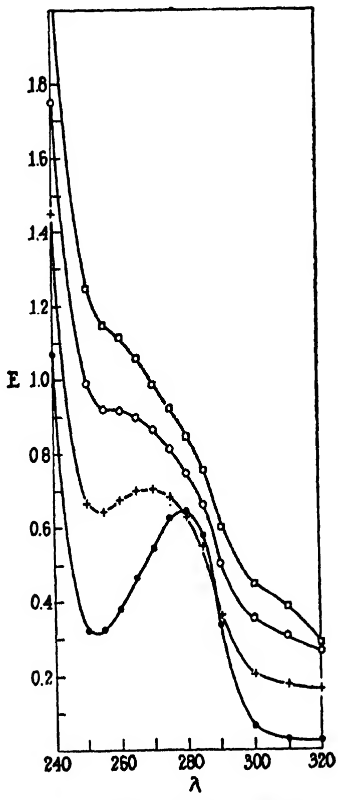


Fig. 1. Absorption spectra of bovine serum albumin before and after oxidation with 0.03 M $LiIO_4$.

● = native bovine albumin.
+ = bovine albumin oxidized 24 hours with $LiIO_4$.
○ = " " " 48 " " "
□ = " " " 72 " " "

absorption below 280 mμ is not only greater, but there is a shift of 10 mμ in the maximum. At the end of 48 and 72 hours contact with the reagent the absorption becomes progressively greater throughout the entire range of wave lengths, and there is no discernible peak. This shift in absorption spectrum is comparable to that shown by the Type III pneumococcus immune globulin after oxidation with periodate.

Determination of Amino Acids.—Because of the shift and final disappearance in the point of maximum absorption of serum albumin following oxidation with periodate, it was thought advisable to analyze the oxidized protein for its tyrosine, tryptophane, cystine, and cysteine content.

Cystine and Cysteine.—Hydrolysates (2) of the native and oxidized proteins were analyzed for their content of cystine and cysteine (3). The values are recorded in Table I. It can be seen that the cystine content of the native protein was essentially the same as that reported by other investigators (8); the cysteine content, on the other hand, was considerably lower. Repeated analyses confirmed this observation and the difference probably resides in the fact that our particular sample of bovine serum albumin had actually a lower content of labile -SH groups.

When cystine and cysteine determinations were carried out on hydrolysates of the protein previously oxidized for 72 hours, the two values were considerably lower, 2.45 and 0.13 per cent, respectively. This would indicate that a marked decrease in S-S and in -SH groupings had taken place, without a comparable loss in total sulfur, for it can be seen in Table I that the total sulfur content of the native and oxidized protein was found to be 1.90 and 1.68 per cent, respectively.

Tyrosine.—A sample of native and of oxidized bovine serum albumin dissolved in 5 N NaOH containing 20 mg. of protein per ml. was hydrolyzed in a sealed tube for 24 hours at 100° (4). After removal of tryptophane, or its products of oxidation, as the mercury salt, the tyrosine was determined according to the method of Lugg (5). The native protein was found to contain 5.63 per cent tyrosine. The hydrolysate of the oxidized protein, on the other hand, gave an intensity of color corresponding to 3.28 per cent tyrosine. From the analytical result it appears that approximately 40 per cent of the tyrosine had been destroyed or altered by treatment of the protein with lithium periodate.

Tryptophane.—Both the native and oxidized protein were next analyzed for their tryptophane content (6). The native protein was found to have a tryptophane content of 0.7 per cent, whereas the oxidized protein gave no color with the reagents, indicating that the tryptophane had been destroyed. Since color development, both in the determination of tyrosine and tryptophane, appears to be dependent upon the integrity of the phenolic and indole rings, it can be assumed that the latter have in each instance suffered chemical degradation, the extent of which is unknown.

In this connection it might be said that when pure tyrosine was subjected to the action of 0.03 M $LiIO_4$ at pH 7.2, approximately 1 mol of the reagent was consumed over a period of 8 hours. During the course of the oxidation the solution darkened and eventually deposited a precipitate. Chromatographic adsorption of the reaction mixture on activated alumina revealed at least four bands, indicating that a complex series of chemical reactions had taken place.

Electrophoretic Properties of Native and Oxidized Bovine Serum Albumin.—In

the foregoing account it has been shown that the oxidation of bovine serum albumin with lithium periodate causes marked changes in the chemical and physical properties of the protein. It seemed of interest, therefore, to investigate the electrophoretic behavior of the protein after treatment with this reagent.

TABLE I

*Analysis of Native and Oxidized Bovine Serum Albumin**

Analysis	Native bovine albumin	Bovine albumin after 72 hrs. oxidation with 0.033 M $LiIO_4$
$[\alpha]_D^{22°}$	−53.0°	−69.0°
Total N, *per cent*	16.00	14.51
Total S, " "	1.91	1.68
Cystine, " "	5.65	2.45
Cysteine, " "	0.43	0.13
Tyrosine, " "	5.63	3.28
Tryptophane, *per cent*	0.70	0.00

* Oxidized 72 hours with 0.033 M $LiIO_4$.

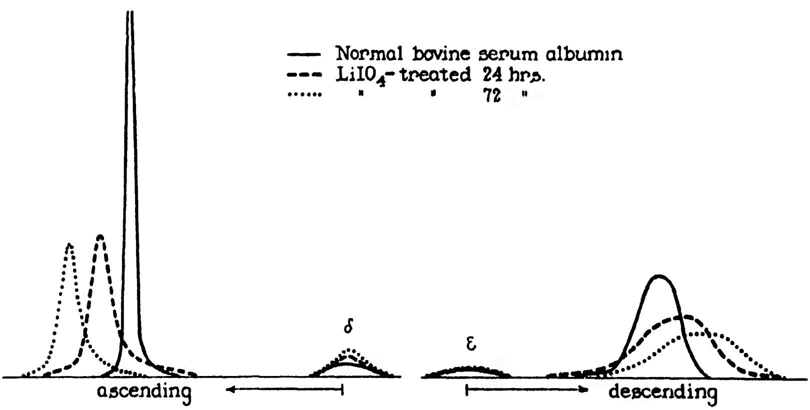


FIG. 2. Electrophoretic patterns of bovine serum albumin before and after treatment with 0.03 M lithium periodate.

In the experiment recorded in Fig. 2 the electrophoresis of bovine serum albumin before and after treatment with lithium periodate was carried out in a sodium phosphate buffer at pH 7.7, an ionic strength 0.1, at a potential gradient of 6.34 volts per cm. for 9,000 seconds. The full curve represents native bovine serum albumin, the dashed and dotted curves are patterns of the same preparation after oxidation with 0.03 M lithium periodate for 24 and 72 hours, respectively. From the figure it is apparent that a qualitative difference in the patterns exists; the boundaries of the two oxidized proteins are more

diffuse than is that of the native material. Under experimental conditions in which time and potential gradient have been kept constant, superimposition of the electrophoretic patterns permits a direct comparison of the approximate mobilities of the three protein preparations. Thus it can be seen that the mobilities of the substances are different. The actual values of $u = -6.77 \times 10^{-5}$ and $u = -7.33 \times 10^{-5}$ for the samples treated for 24 and 72 hours, respectively, are considerably higher than that of $u = -6.14 \times 10^{-5}$ of the native protein.

The large boundary spreading shown by the two oxidized proteins suggests that these preparations are less homogeneous than the native albumin. It seemed advisable, therefore, to extend the electrophoretic study over a wider range of pH values. It was observed that the protein oxidized for 72 hours migrated as a single peak at all pH values studied, except in one instance where the determination was carried out in a 0.1 N sodium acetate buffer at pH 5.64. At this pH a separation of the boundary into two peaks was observed in the ascending patterns. A comparison with bovine serum albumin in the isoelectric pH region was not possible because of the insolubility of the oxidized protein in the pH range of 3.5 to 5.2.

The results of the mobility measurements over a wide pH range of normal bovine serum albumin and of the protein treated with lithium periodate for 72 hours are presented in Table II. All the experiments were carried out in monovalent buffers of 0.1 ionic strength, the composition of which is given in column 1. The mobility values obtained for native bovine serum albumin are in good agreement with those of Longsworth and Jacobsen (9). A comparison of the mobilities of the oxidized protein, column 4, with those of the native protein, column 3, shows that at all pH values below 9 the oxidized material has the more negative mobility; *i.e.*, as a cation it migrates more slowly than the intact protein while as an anion it moves more rapidly.

In an attempt to find further differences between the two proteins, their electrophoretic behavior in a buffer containing methyl orange has also been studied. With the aid of the dialysis experiments and photometric technique of Klotz (10), it was found that the oxidized protein bound but half the quantity of methyl orange as that bound by normal bovine serum albumin. Longsworth and Jacobsen (9) have shown that native bovine serum albumin has a higher mobility in a 0.1 N sodium acetate buffer at pH 5.6 containing 0.0002 M methyl orange than it does in pure acetate buffer. The difference in mobility of the oxidized protein when observed under the same conditions, $\Delta u = 0.3 \times 10^{-5}$, when compared with that of native albumin (9), $\Delta u = 0.6 \times 10^{-5}$, parallels the results obtained in the dialysis experiments.

Antigenicity of Oxidized Plasma Albumin.—In our hands crystalline bovine serum albumin has proved to be a good antigen. Rabbits injected intravenously with 5 mg. of protein for 6 consecutive days, followed by a rest period

TABLE II

Mobilities of Bovine Serum Albumin before and after Treatment with Lithium Periodate in Buffer Solutions of Ionic Strength 0.1

Buffer (1)	pH (2)	$u \times 10^5$ (3)	(4)	Δu (5)
0.1 N HCl — 0.5 N glycine	3.02	7.67	5.4	2.37
0.02 N NaAc — 0.2 N HAc — 0.08 N NaCl	3.62	5.43		
0.02 N NaAc — 0.1 N HAc — 0.08 N NaCl	3.91	4.14		
0.1 N NaAc — 0.1 N HAc	4.64	0.36		
0.1 N NaAc — 0.05 N HAc	4.90	−0.96		
0.1 N NaAc — 0.01 N HAc	5.64	−2.68	−4.68	2.0
0.02 N NaCac — 0.004 N HCac — 0.08 N NaCl	6.76	−4.24	−5.71	−1.47
0.02 N NaV — 0.02 N HV — 0.08 N NaCl	7.84	−6.12	−6.64	−0.62
0.1 N NaV — 0.02 N HV	8.62	−6.64	−6.90	−0.26
0.1 N NaV — 0.005 N HV	9.22	−7.54	−7.53	

Ac = acetate.
Cac = cacodylate.
V = diethylbarbiturate.

TABLE III

Precipitin Reactions in Sera of Rabbits Immunized with Native and Oxidized Bovine Serum Albumin

Serum of rabbit injected with	Test antigen used	Final dilution of test antigen 1:2,000	1:10,000	1:50,000	1:250,000
A	A	++++	++++	+++	+
A_{24}	A_{24}	0	0	+	±
	A	0	0	+	±
A_{72}	A_{72}	0	0	0	0
	A	0	0	0	0

A = native bovine serum albumin.
A_{24} = bovine albumin oxidized for 24 hours with 0.03 M $LiIO_4$.
A_{72} = bovine albumin oxidized for 72 hours with 0.03 M $LiIO_4$.
++++ = complete precipitation with clear supernate.
\+ = very slight precipitation.
0 = no precipitation.

of a week and again injected during 6 days, developed antisera which precipitated strongly the homologous protein. Groups of animals which had received a similar course of immunization with the protein treated with lithium periodate over a 24 and a 72 hour period gave, in the first instance, antisera which were very weak indeed. Those which had received the protein oxidized for 72

hours had no precipitins whatsoever (Table III), nor did they give a detectable immune response even after prolonged injection of the oxidized protein. These observations are contrary to those made with Type III pneumococcus immune globulin (1), where it was found that contact with lithium periodate for 24 hours did not destroy antigenicity.

TABLE IV

Precipitin Reactions of Oxidized Bovine Serum Albumin in Serum of a Rabbit Immunized with Native Albumin

Test antigen used	Final dilution of test antigen			
	1:2,000	1:10,000	1:50,000	1:250,000
A	++++	++++	+++	+
A_{24}	++++	+++	++	+
A_{48}	++	+±	+	±
A_{72}	++	+±	+	0

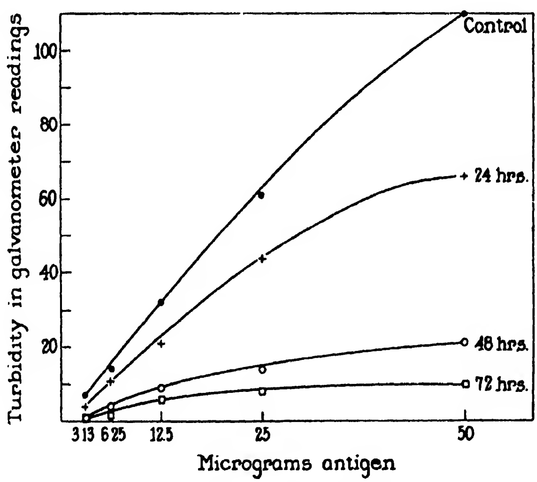


FIG. 3. Turbidimetric precipitin reaction of oxidized bovine albumin in native albumin antiserum.

Not only did the oxidized albumin fail to elicit homologous antibodies in rabbits, as can be seen in Table III, but there were no antibodies present in these sera capable of precipitating the native unaltered protein. This would indicate that no intact native protein molecules remain after the albumin has been treated with lithium periodate. It is of considerable interest, however, to note that the treated albumin is still capable of precipitating some of the antibodies in the sera of rabbits immunized with native serum albumin (Table

IV). When a quantitative turbidimetric estimation (11) of these reactions was made, it was observed (Fig. 3) that the intensity of the reaction diminished the longer the albumin fraction remained in contact with the reagent. From the results of these experiments it appears justified to conclude that the ability of serum albumin to incite antibody formation in rabbits is destroyed when the protein is subjected to the action of lithium periodate. The reason for this probably resides in the oxidative changes which take place in certain of the amino acids; these changes are not so profound, however, as to impair the ability of the altered protein to combine with the antibodies specifically directed toward the intact native protein molecule.

DISCUSSION

Periodic acid is not a benign reagent but a vigorous oxidant capable of bringing about extensive chemical changes in many types of organic compounds (12). Certain monosaccharides in acid solution are rapidly and completely broken down by the reagent; polysaccharides of appropriate structure suffer a severance of the component monosaccharide molecules with the formation of polyaldehydes and other ill defined products of oxidation. In addition, amino acids of certain types are readily oxidized by periodic acid. It is not surprising, therefore, that proteins may also undergo chemical alteration when brought in contact with the reagent.

That bovine serum albumin suffers irreparable damage on prolonged contact with lithium periodate is apparent from the results presented above. The chemical changes involve a slight loss in total nitrogen, a change in specific optical rotation, and an alteration or possible destruction of all or part of certain amino acids. The total sulfur content of the protein is somewhat diminished, yet the cystine, cysteine, and tyrosine content, the measure of which is based upon color development which in turn is dependent upon a specific chemical configuration, are considerably diminished. The tryptophane appears to be completely destroyed. That these chemical alterations are progressive and are a function of time is apparent from the gradual changes which occur in the absorption spectrum, and in electrophoretic behavior of the protein. Yet these changes are not so great as to cause a loss of properties which would no longer permit the material to be classified as protein. Solutions of the product of oxidation are still precipitated by high concentrations of salts; they give positive biuret tests, are precipitable by trichloracetic acid, and are hydrolyzed by crystalline trypsin. In some respects the oxidized material resembles a denatured protein, —in absorption spectrum, in solubility in high concentration of salts, and in precipitability at acid pH values. Unlike denatured protein, the oxidized material is incapable of eliciting antibodies in rabbits. This loss of function is believed to be associated with alterations in the aromatic amino acid content, and in this respect the material is analogous to gelatin (13)

which contains neither tyrosine nor tryptophane, and is not antigenic. That the oxidized protein is still specifically precipitated by antibodies to native albumin, can best be explained on the basis that not all those groups of the protein which are responsible for antigen-antibody combination have been destroyed, even after prolonged contact with the reagent.

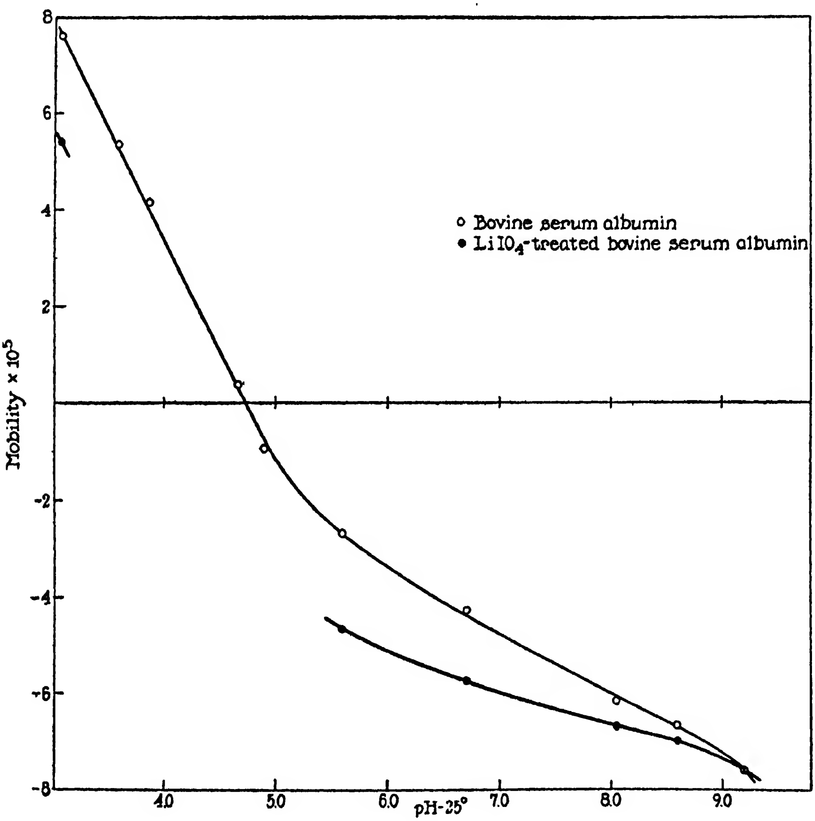


FIG. 4. Mobilities of normal and oxidized bovine serum albumin as a function of pH.

Because of the limited solubility of the oxidized protein, the mobility measurements recorded in this paper were necessarily confined to pH 3.0 and to the pH range of 5.6 to 9.2. A determination of the isoelectric point of the periodate-treated protein was therefore not possible. If the mobility data presented in Table II are plotted as ordinate against pH as abscissae, Fig. 4, it is apparent that the oxidized bovine serum albumin has a higher negative net charge than the normal bovine serum albumin at any given pH. This would indicate that the isoelectric point of the oxidized protein is at a pH more acid than that of

the native albumin and might possibly be explained by the destruction of the imidazole ring of the histidine molecule. The results of this report, however, do not permit of any extensive discussion concerning the groups which may have been altered during the oxidative process and which contribute to the electrophoretic behavior of this protein.

SUMMARY

A study of the chemical, physicochemical, and immunological changes in bovine serum albumin, brought about by oxidation with lithium periodate, has been made. It has been shown that destruction of certain amino acids occurs, that a change in the absorption spectrum takes place, and that the electrophoretic behavior of the protein is altered. Prolonged contact of bovine albumin with lithium periodate destroys its ability to incite antibodies in experimental animals.

BIBLIOGRAPHY

1. Goebel, W. F., Olitsky, P. K., and Saenz, A. C., *J. Exp. Med.*, 1948, **87**, 445.
2. Kassell, B., and Brand, E., *J. Biol. Chem.*, 1938, **125**, 115.
3. Brand, E., and Kassell, B., *J. Gen. Physiol.*, 1941, **25**, 167.
4. Brand, E., and Kassell, B., *J. Biol. Chem.*, 1939, **131**, 489.
5. Lugg, J., *Biochem. J.*, 1937, **31**, 1431.
6. Sullivan, M. X., Milone, H. S., and Everitt, E. L., *J. Biol. Chem.*, 1938, **125**, 471.
7. Longsworth, L. G., *Chem. Rev.*, 1942, **30**, 323; *Ind. and Eng. Chem., Analytical Edition*, 1946, **18**, 219.
8. Brand, E., Kassell, B., and Saidel, L. J., *J. Clin. Inv.*, 1944, **23**, 437.
9. Longsworth, L. G., and Jacobsen, C. F., *J. Physic. and Coll. Chem.*, 1949, **53**, 126.
10. Klotz, I. M., Walker, F. M., and Pivan, R. B., *J. Am. Chem. Soc.*, 1946, **68**, 1486.
11. Libby, R. L., *J. Immunol.*, 1938, **34**, 269; 1938, **35**, 289.
12. Adams, R., Organic Reactions, New York, John Wiley and Sons, Inc., 1944, **2**, 341.
13. Landsteiner, K., The Specificity of Serological Reactions, Cambridge, Harvard University Press, 1945, 62.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, May 1, 1949, Vol. 89, No. 5, pp. 491–505]

THE MECHANISM OF ACTIVE CEREBRAL IMMUNITY TO EQUINE ENCEPHALOMYELITIS VIRUS

I. INFLUENCE OF THE RATE OF VIRAL MULTIPLICATION

BY R. WALTER SCHLESINGER,* M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research, and the Division of Infectious Diseases, The Public Health Research Institute of The City of New York, Inc.*)

(Received for publication, January 11, 1949)

It has been found (1) that mice vaccinated with inactivated Western equine encephalomyelitis (W.E.E.) virus were less resistant to intracerebral challenge doses of the "R.I." strain than to comparable doses of other strains of W.E.E. virus. This observation could not be ascribed to antigenic or serologic differences between the R.I. and other strains, nor to differences in immunizing potency, since immunization with the R.I. strain protected mice more effectively against heterologous strains of W.E.E. virus than against the homologus strain. The latter had been subjected, over a period of many years, to many brain-to-brain passages in mice, and in the course of this treatment its lethal titer had increased and the survival time of inoculated mice was considerably shortened. It seemed most likely that the difficulty in protecting mice against the R.I. strain was connected with its ability to kill mice more rapidly. The hope seemed warranted that further comparative studies on the properties of this and one of the other strains would lead to a better understanding of factors involved in the mechanism of immunity to neurotropic viruses. The present paper deals in the main with studies on the comparative growth rates in the mouse brain of the R.I. and another strain of W.E.E. virus. In addition, further experiments will be described which illustrate the effect of strain differences on the reaction of immunized animals to intracerebral inoculation. The host factors which are responsible for variations in the response will be discussed in a subsequent paper (2).

Materials and Methods

Virus Strains.—The 2 strains of W.E.E. virus used were the R.I. strain and the Kelser strain previously described by Olitsky, Morgan, and Schlesinger (1). They differed in the manner already mentioned; *i.e.*, the R.I. strain had a higher lethal titer and, after intracerebral inoculation of comparable amounts, killed mice about twice as rapidly as the Kelser strain. The R.I. strain has been carried through many brain-to-brain passages in mice since 1933, while the Kelser strain has been subjected to only a few such transfers. The exact number of passages has not been recorded for either strain.

* Present address: The Public Health Research Institute of The City of New York, Inc.

Stock Virus Suspensions.—Brains were harvested from mice inoculated intracerebrally with about 100 LD_{50} of virus which were sacrificed when encephalitic signs were first seen. Crude 20 per cent homogenates of such brains were prepared in inactivated (56°C. for 30 minutes) normal rabbit serum diluted 1:2 in saline. The suspensions were kept frozen in sealed glass ampules in the dry ice chest. A fresh ampule was used for each experiment.

TABLE I

W.E.E. Virus, R.I. Strain: Frequency and Standard Deviations of Intracerebral Titers in Mice

Titer (X)*	Frequency (f) of titer X in sample				$f(X - \bar{X})^2$			
	A	B	C	Total	A	B	C	Total
8.5			1	1	0.00	0.00	0.25	0.49
8.6	1	1		2	0.36	0.36	0.00	0.72
8.7		1	1	2	0.00	0.25	0.09	0.50
8.8	2	1		3	0.32	0.16	0.00	0.48
8.9				0	0.00	0.00	0.00	0.00
9.0	6	1	2	9	0.24	0.04	0.00	0.36
9.1				0	0.00	0.00	0.00	0.00
9.2	1	1	1	3	0.00	0.00	0.04	0.00
9.3	4	2		6	0.04	0.02	0.00	0.06
9.4		1		1	0.00	0.04	0.00	0.04
9.5	3	3	1	7	0.27	0.27	0.25	0.63
9.6		1		1	0.00	0.16	0.00	0.16
9.7	1			1	0.25	0.00	0.00	0.25
9.8				0	0.00	0.00	0.00	0.00
9.9				0	0.00	0.00	0.00	0.00
10.0	1			1	0.04	0.00	0.00	0.04
No. of tests (N)	19	12	6	37	—	—	—	—
Mean titer ($\bar{X}$)	9.2	9.2	9.0	9.2	—	—	—	—
$\Sigma (X - \bar{X})^2$	—	—	—	—	2.12	1.30	0.63	4.33
S.D. (σ) $= \sqrt{\frac{\Sigma(X - \bar{X})^2}{N-1}}$	—	—	—	—	0.343	0.344	0.355	0.347

* Titers are expressed as log LD_{50}/0.03 gm. of brain tissue.

All dilutions of stock virus were made in saline containing 10 per cent inactivated rabbit serum. The intracerebral inoculum was 0.03 ml. per mouse.

Standardization of Stock Viruses.—Titers of the stock virus suspensions have been calculated according to the 50 per cent end-point method of Reed and Muench (3) and expressed in terms of LD_{50} per 0.03 gm. of brain tissue.

With one exception, all the experiments to be described in this and the following paper (2) were done with a limited number of stock samples of the two strains. In Tables I and II are given the results of multiple titrations of these samples in mice and summaries of the cal-

culations of the standard deviations according to the formula previously used for other viruses by Lauffer and Miller (4) and by Horsfall and Curnen (5). It will be seen that the values found for individual samples were almost identical. Therefore, these values were combined, giving for the R.I. strain a mean titer of $10^{9.2}$ with a standard deviation of 0.347 log, and for the Kelser strain a mean titer of $10^{7.5}$ with a standard deviation of 0.345 log.

This standardization covers only the reproducibility of end-points on stock samples but not necessarily variables and sampling errors involved in determinations of the growth rate.

TABLE II

W.E.E. Virus, Kelser Strain: Frequency and Standard Deviations of Intracerebral Titers in Mice

Titer (X)*	Frequency (f) of titer (X) in sample			$f(X - \bar{X})^2$		
	A	B	Total	A	B	Total
7.0	1	1	2	0.25	0.16	0.50
7.1						
7.2	2	2	4	0.18	0.08	0.36
7.3	1		1	0.04		0.04
7.4						
7.5		3	3		0.03	0.00
7.6	2		2	0.02		0.02
7.7	1	1	2	0.04	0.09	0.08
7.8						
7.9		1	1		0.25	0.16
8.0	1	1	2	0.25	0.36	0.50
8.1	1		1	0.36		0.36
Total No. tests (N)............	9	9	18	—	—	—
Mean titer ($\bar{X}$)................	7.5	7.4	7.5	—	—	—
$\Sigma (X - \bar{X})^2$....................	—	—	—	1.14	0.97	2.02
S.D. (σ) = $\sqrt{\frac{\Sigma(X - \bar{X})^2}{N - 1}}$	—	—	—	0.377	0.348	0.345

* Titers are expressed as log LD_{50}/0.03 gm. of brain tissue.

Technique of the Experiments on Virus Multiplication.—Mice inoculated intracerebrally with various amounts of virus were sacrificed at appropriate intervals. Their brains were harvested and stored in the dry ice chest either whole or as homogenized suspensions in saline containing 10 per cent inactivated normal rabbit serum. In most experiments, pools of 2 or 3 brains were prepared for each interval. At the time of test, as a rule, 3.2-fold serial dilutions of the uncentrifuged brain homogenates were made over the suitable range, and at least 3 or 4 mice were inoculated intracerebrally with each dilution. For convenience, the 3.2-fold steps were considered as equal to 0.5 log-fold, and titers were calculated on that basis according to the method of Reed and Muench (3).

Vaccines.—Mice inoculated intracerebrally with about 100 LD_{50} of R.I. stock virus were

chloroformed after 40 hours when they began to show signs of encephalitis. Under deep anesthesia, the thoracic cavity was opened, the heart cut through, and the blood was drained onto absorbent cotton covered by gauze. The brains were removed, and excessive blood was washed off with saline. 4 ml. of cold saline was added per brain to make a 10 per cent suspension which was homogenized for 1 minute in a chilled Waring blendor. It was then filtered through several layers of gauze, and finally formalin was added to a final concentration of 0.3 per cent. The vaccine was shaken daily and kept in a refrigerator. It was used when proved non-infectious by intracerebral test in mice.

As a rule, immunization consisted of 6 intraperitoneal doses given in 2 courses of 3 daily injections 1 week apart. The stock vaccine was diluted in saline and the inoculum was 0.3 ml. The total amount of vaccine given is stated in the text in terms of equivalent amounts of undiluted vaccine. Challenge inoculations were given 2 weeks after the first dose of vaccine.

Mice.—Albino Swiss mice, weighing 7 to 10 gm., were obtained from 2 dealers. There was no difference between them in response to any of the procedures used.

EXPERIMENTAL

Comparative Rates of Multiplication of the R.I. and Kelser Strains of W.E.E. Virus

(*a*) *The Initial Yield of Virus in Relation to the Amount Inoculated.*—A finding common to all the present experiments was an initial decrease in the amount of virus recoverable from infected brains. Inoculation of a volume of 0.03 ml. into a brain weighing 0.4 gm. would result in 13.3-fold dilution. Hence, after inoculation of $10^n LD_{50}$, the expected yield prior to viral multiplication would be $10^{(n-1.12)}$ LD_{50} per 0.03 gm. of brain tissue. Actually, the mean yield at 1 hour after inoculation was only 3.5 per cent of the expected recovery in the case of the R.I. strain and 10.2 per cent in that of the Kelser strain. The disappearance from the brain of 90 to 96 per cent of the inoculum was independent of the amount of virus given, as shown in Tables III and IV. The significance of this loss is not clearly understood,[1] and for the purpose of studying the viral growth rate the extent of the loss is important mainly because it establishes the true starting point of multiplication.

(*b*) *Correlation between Rate of Virus Increase and Course of Disease in Mice.*[2]—Of 45 mice inoculated intracerebrally with $10^{2.6}$ LD_{50} of the R.I. strain, 30 were sacrificed in pairs at 2 to 4 hour intervals. Definite signs of disease, *i.e.* spontaneous convulsive seizures or continuous circling, were first seen 38 hours after inoculation. All remaining mice died after 42 to 48 hours. 64 mice received intracerebrally $10^{3.4}$ LD_{50} of the Kelser strain, and of these 48 were sacrificed in pairs at 4 hour intervals. Here, definite encephalitic signs began 74 hours after inoculation and the survival time of the remaining mice varied from 3 to 7 days with an average of 3.7 days.

---

[1] Losses of similar magnitude were encountered when *E. coli* phages T1 or T7 were inoculated intracerebrally in mice.

[2] The samples of the 2 strains of virus used in this test have not been included in Tables I and II.

The rates of multiplication of the 2 strains are presented in Fig. 1. The titer of the R. I. strain rose logarithmically up to 32 hours after inoculation. By that time, its increase was 1,000 times greater than that of the Kelser strain. The R.I. strain reached its maximum titer about 6 hours before onset of definite encephalitic signs. The growth rate of the Kelser strain appeared to slow

TABLE III

W.E.E. Virus, R.I. Strain: Difference between Expected and Actual Yield of Virus from Mouse Brains 1 Hour after Intracerebral Inoculation

Test No.	Inoculum (a)*	Yield at 1 hr. Expected = (a) − 1.12 (b)*	Yield at 1 hr. Actual (c)*	Difference (b) − (c)*	Deviation from mean difference (d)	$(d)^2$
1	3.2	2.08	1.12	0.96	−0.49	0.240
2	3.7	2.58	0.70	1.88	+0.43	0.185
3	3.7	2.58	0.95	1.63	+0.18	0.032
4	4.2	3.08	1.5	1.58	+0.13	0.017
5	4.2	3.08	1.0	2.08	+0.63	0.397
6	4.2	3.08	1.5	1.58	+0.13	0.017
7	4.2	3.08	1.7	1.38	−0.07	0.049
8	5.2	4.08	3.0	1.08	−0.37	0.137
9	5.2	4.08	2.73	1.35	−0.10	0.010
10	7.2	6.08	4.75	1.33	−0.12	0.014
11	7.2	6.08	4.88	1.20	−0.25	0.063
12	8.2	7.08	5.95	1.13	−0.32	0.102
13	8.2	7.08	5.37	1.71	+0.26	0.068
Total (Σ)..........	68.6	54.04	35.15	18.89	3.48	1.331
Mean $\frac{\Sigma}{N}$............	5.277	4.157	2.704	1.453	0.268	—

$$\text{Standard deviation } (\sigma) = \sqrt{\frac{\Sigma(d^2)}{N-1}} = \sqrt{\frac{1.331}{12}} = 0.333$$

* All figures given as log LD_{50}.

down beginning about 36 hours after inoculation and came to a standstill after about 62 hours or 12 hours before onset of definite encephalitic signs.

Thus, the difference between the 2 strains with regard to incubation and survival periods reflected a difference of similar magnitude between their rates of multiplication.

(*c*) *Latent Phase Preceding Multiplication of the R.I. and the Kelser Strains.*—As is shown in Fig. 2, no viral growth was demonstrated for about 3 hours in the brains of mice inoculated with $10^{3.7}$ LD_{50} of the R.I. strain. In the case of the Kelser strain, after inoculation of $10^{4.5}$ LD_{50}, there was no significant multiplication for 5 hours (Fig. 3). After these latent periods, there was

growth at a fairly constant rate of the R.I. strain, while that of the Kelser strain appeared to come to a standstill between 7 and 10 hours after inoclation. Between 3 and 10 hours after inoculation, there was a 1,000-fold increase in titer of the R.I. strain, and only a 10-fold increase in that of the Kelser strain.

(*d*) *Influence of Variations in the Size of the Inoculum on the Rate of Viral Multiplication.*—After inoculation of various amounts of W.E.E. virus in excess of 100 LD_{50} there was relatively little variation in the survival time

TABLE IV

W.E.E. Virus, Kelser Strain: Difference between Expected and Actual Yield of Virus from Mouse Brains 1 Hour after Intracerebral Inoculation

Test No.	Inoculum (*a*)*	Yield at 1 hr. Expected = (*a*) − 1.12 (*b*)*	Yield at 1 hr. Actual (*c*)*	Difference (*b*) − (*c*)*	Deviation from mean difference (*d*)	$(d)^2$
1	2.5	1.38	<0.70	>0.68	>0.31	0.096
2	2.5	1.38	<0.50	>0.88	>0.11	0.012
3	4.0	2.88	2.50	0.38	−0.61	0.372
4	4.0	2.88	2.50	0.38	−0.61	0.372
5	4.0	2.88	1.25	1.63	+0.64	0.409
6	4.5	3.38	2.62	0.76	−0.23	0.053
7	4.5	3.38	2.38	1.00	+0.01	0.0001
8	4.5	3.38	2.50	0.88	−0.11	0.012
9	4.5	3.38	1.66	1.72	+0.73	0.533
10	4.5	3.38	1.91	1.47	+0.48	0.230
11	6.5	5.38	4.12	1.26	+0.27	0.073
12	6.5	5.38	4.50	0.88	−0.11	0.012
Total (Σ)..........	52.5	39.06	27.14	11.92	4.22	2.173
Mean $\frac{\Sigma}{N}$...........	4.375	3.255	2.261	0.993	0.352	—

$$\text{Standard deviation } (\sigma) = \sqrt{\frac{\Sigma(d^2)}{N-1}} = \sqrt{\frac{2.173}{11}} = 0.444$$

* All figures given as log LD_{50}.

of mice (1). This similarity in the course of the disease is matched by the observation that the growth curves after inoculation of different amounts tended to converge so that the maximum titer was reached at about the same time regardless of the size of the inoculum. This is illustrated for the 2 strains in Figs. 4 and 5. It will also be seen that after inoculation of relatively small amounts of the R.I. strain (see Fig. 4), there was a tendency for the growth rates to parallel each other.

On the basis of these findings regarding the growth rates of the 2 strains,

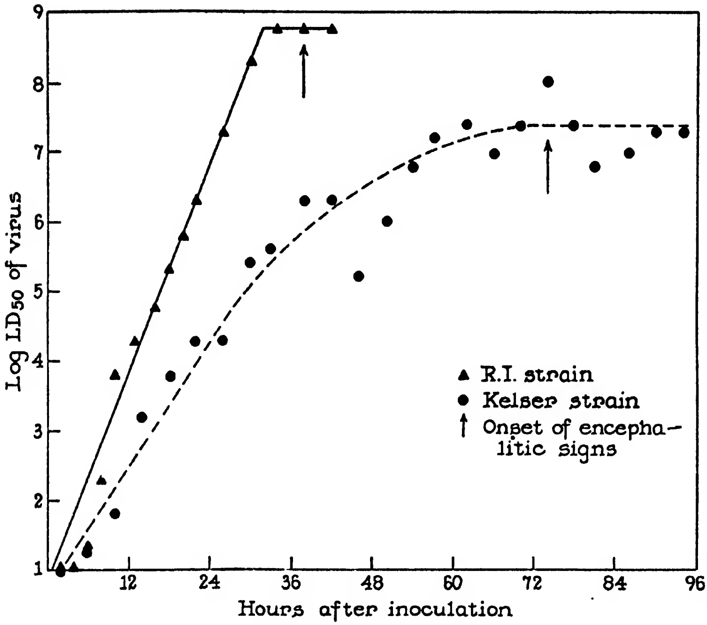


FIG. 1. Comparative rates of multiplication of R.I. and Kelser strains of W.E.E. virus in brains of normal mice.

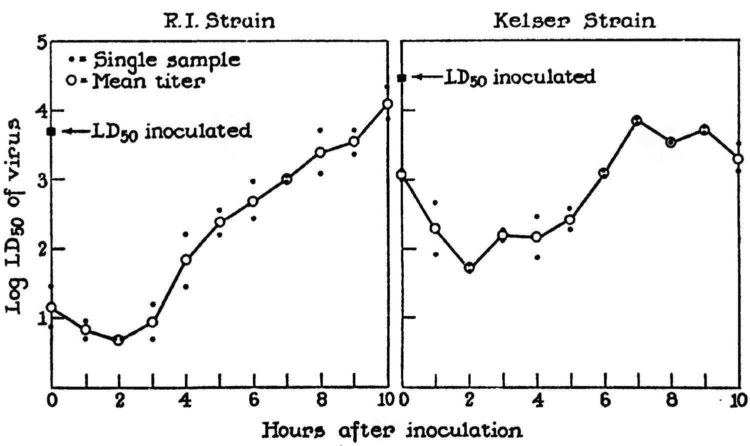


FIG. 2 FIG. 3

FIGS. 2 and 3. Rate of multiplication of W.E.E. virus in brains of mice after intracerebral inoculation.

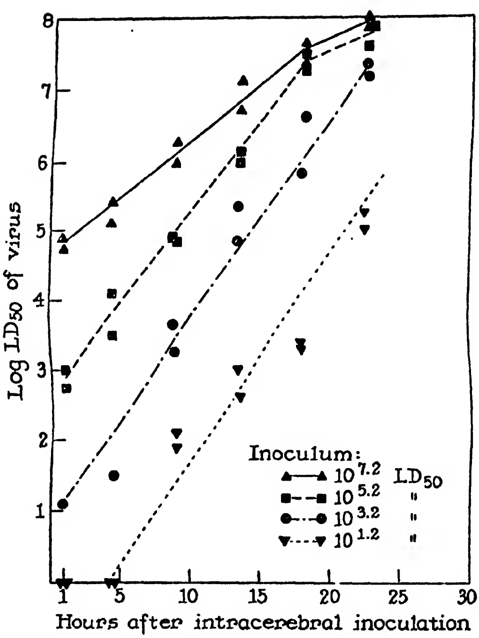


FIG. 4. Rates of multiplication of W.E.E. virus, R.I. strain, after intracerebral inoculation of different amounts.

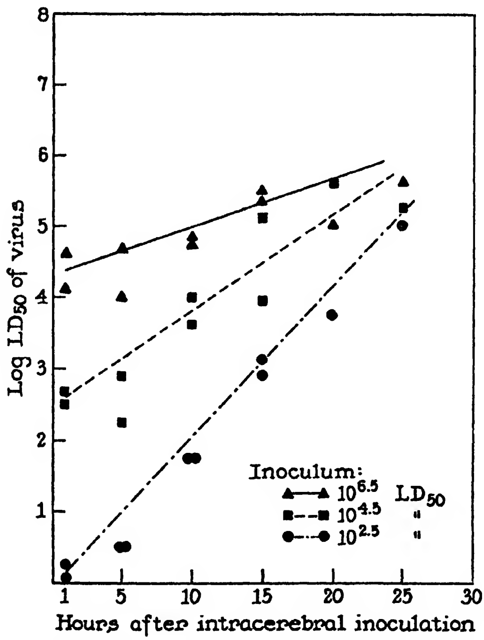


FIG. 5. Rates of multiplication of W.E.E. virus, Kelser strain, passage 3, after intracerebral inoculation of different amounts.

the difference in response to them of immunized mice was reexamined. The primary purpose of the experiments to be described in the following sections was to define this difference quantitatively and to establish a baseline for an investigation into its immediate cause.

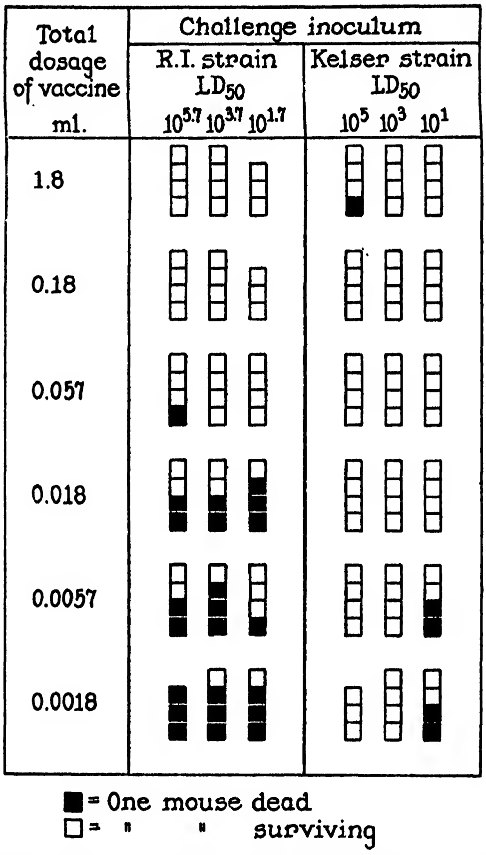


FIG. 6. Comparative degrees of resistance of immunized mice to intracerebral challenge doses of two strains of W.E.E. virus.

The Response of Immunized Mice to Intracerebral Challenge Doses of the R.I. and Kelser Strains

Fig. 6 presents the results of an experiment in which mice immunized with different amounts of vaccine were challenged by the intracerebral route with comparable graded amounts of the R.I. or the Kelser strain of W.E.E. virus. It is clear that after immunization with relatively small doses of vaccine (less than 0.057 ml.) there was a much greater degree of resistance to the Kelser

than to the R.I. strain. This difference in response was masked when a larger dosage of vaccine was used.[3]

It is noteworthy that four of eight mice immunized with the smallest amounts of vaccine (0.0057 and 0.0018 ml.) succumbed to a challenge dose of 10 LD_{50} of the Kelser strain while all the mice challenged with 10^3 or 10^5 LD_{50} survived. This "paradoxical" type of response was found to be characteristic of mice with low degree of immunity and will be discussed in a subsequent paper (2).

TABLE V

Comparison of Typical Titrations in Mice of Three Representative Samples of W.E.E. Virus

Sample of virus	Dilution of virus	Day of death: Individual mice	Day of death: Average	Total mortality	Log LD_{50}
R.I. strain	10^{-7}	2, 2, 2, 2	2.0	4/4	9.2
	10^{-8}	3, 3, 4, 5	3.75	4/4	
	10^{-9}	3, 3, 0, 0	3.0	2/4	
	10^{-10}	4, 0, 0, 0	(4)	1/4	
Kelser strain passage 3	10^{-1}	6, 7, 7, 7, 8	7.0	5/5	>7.4
	10^{-2}	6, 7, 7, 7, 9	7.2	5/5	
	10^{-3}	6, 6, 6, 7, 9	6.8	5/5	
	10^{-4}	5, 7, 8, 8, 9	7.4	5/5	
	10^{-5}	6, 6, 7, 8, 9	7.2	5/5	
	10^{-6}	5, 6, 6, 7, 8	6.4	5/5	
	10^{-7}	5, 6, 8, 8, 0	6.75	4/5	
Kelser strain passage 40	10^{-6}	3, 3, 3, 3, 7	3.8	5/5	7.6
	10^{-7}	3, 3, 3, 4, 6	3.8	5/5	
	10^{-8}	4, 0, 0, 0, 0	(4)	1/5	
	10^{-9}	0, 0, 0, 0, 0	—	0/5	

Effect of Continued Brain-to-Brain Passages on the Behavior of the Kelser Strain

In an effort to show that the distinct properties of the R.I. strain had resulted from its continued propagation in the mouse brain, the slower Kelser strain was subjected to a rapid succession of brain-to-brain passages in mice.

After the virus had undergone a total of 40 passages in this laboratory,

[3] In earlier experiments (1), the difference between strains was demonstrable even in mice immunized with relatively large doses of vaccine. At that time, vaccines had been prepared from infected chick embryo and had been centrifuged. They were less potent than the ones used more recently. Studies on St. Louis and Japanese B encephalitis vaccines have shown that centrifugation may result in considerable loss of antigenic potency (6). The present studies, carried out with crude vaccines prepared from infected mouse brain, confirm the report of Ruchman (7) to the effect that there is no difficulty in effectively immunizing mice against the R.I. strain.

TABLE VI

Comparative Neutralization Test with Mouse Immune Serum against the R.I. Strain and Two Variants of the Kelser Strain of W.E.E. Virus

Log final dilution of serum	R.I. strain				Kelser passage 3				Kelser passage 40			
	LD_{50} in mixture			Neutralization index*	LD_{50} in mixture			Neutralization index	LD_{50} in mixture			Neutralization index
	$10^{3.2}$	$10^{2.2}$	$10^{1.2}$		10^{3}	10^{2}	10^{1}		$10^{3.2}$	$10^{2.2}$	$10^{1.2}$	
0.8	1/4‡	0/4	0/4	>3.53	1/4	0/4	0/4	>3.33	2/4	0/4	0/4	3.2
1.8	4/4	4/4	0/4	1.7	3/4	1/4	1/4	2.33	4/4	0/4	1/4	2.58
2.3	4/4	3/4	0/4	1.87	4/4	4/4	1/4	1.33	4/4	3/4	0/4	1.87
2.8	4/4	3/4	2/4	1.4	3/3	3/3	1/4	1.33	4/4	4/4	2/4	1.2
3.3	4/4	3/4	2/4	1.4	4/4	3/4	2/4	1.2	4/4	4/4	0/4	1.7
3.8	4/4	4/4	2/4	1.2	4/4	4/4	3/4	<0.7	4/4	4/4	4/4	<0.7
Neutralizing titer§	1.13	1.74	3.3	—	1.3	2.04	3.1	—	0.8	1.83	3.36	—

* Neutralization index, log LD_{50} of virus neutralized by indicated dilution of serum.

‡ Numerator, number of mice dead. Denominator, number inoculated.

§ Neutralizing titer, log of estimated highest dilution of serum which would protect 50 per cent of the mice in mixture with indicated amount of virus.

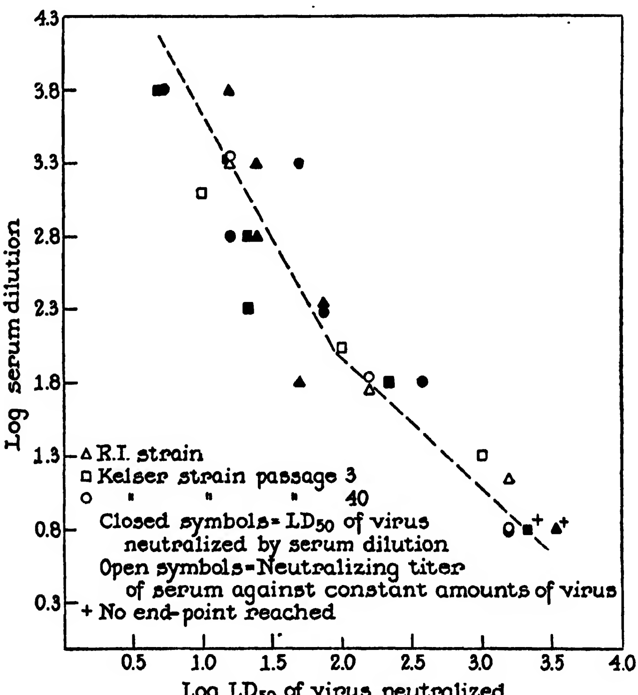


FIG. 7. Neutralization of W.E.E. virus by hyperimmune mouse serum.

convulsive seizures were regularly seen in all mice 2 days after inoculation of 10^{-2} diluted brain tissue. In Table V, typical titrations of the R.I. strain and of 3rd and 40th passage Kelser virus are presented. Although the titer of the 40th passage virus was still relatively low, the average survival period of mice infected with it had so decreased as to approach that of mice infected with the R.I. strain.

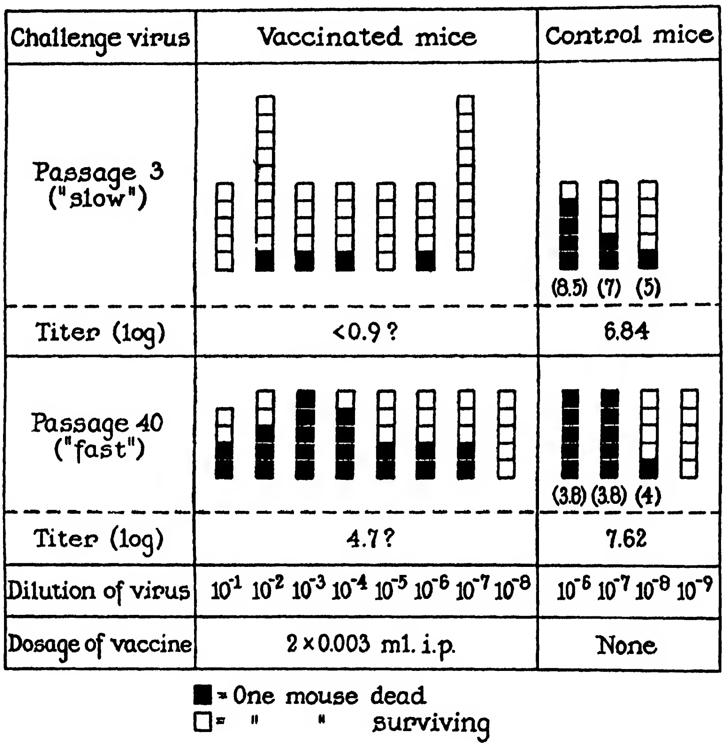


FIG. 8. Comparative degrees of resistance of immunized mice to intracerebral challenge doses of "slow" and "fast" variants of the Kelser strain.

Numbers in parentheses indicate the average survival periods in days.

The same three virus preparations were employed in a neutralization test for the purpose of establishing their serological identity.

Serial 0.5 log-fold dilutions of a W.E.E. R.I. immune mouse serum were mixed with approximately 10, 100, and 1,000 LD_{50} of each of the 3 virus samples. After 2 hours' incubation in the 37°C. water bath, the mixtures were injected intracerebrally into mice. The results of this test are given in Table VI. The data are summarized in Fig. 7 which shows that the 3 samples were neutralized equally well by the serum. Thus, no measurable serological changes

resulted from continued propagation of either strain of W.E.E. virus in the mouse brain.

It remained to be seen whether the "fast" derivative of the Kelser strain differed from its parent strain when used as challenge inoculum in immunized mice. After immunization with 0.006 ml. of vaccine, mice were challenged intracerebrally with graded doses of passage 3 or passage 40 samples of the Kelser strain. The outcome of this test is presented in Fig. 8. After 37 additional passages, the Kelser strain behaved like the R.I. strain in that mice vaccinated to an extent such that they resisted almost without exception even maximal amounts of the "slow" strain were only slightly protected against its "fast" derivative. In the latter group, deaths were scattered over the entire range of virus dilutions. This illustrates the inability to express intermediate degrees of immunity in mathematical terms. With the small number of animals employed, it appeared that the chance of survival was hardly greater after challenge with 4 than with 4 million LD_{50}.

DISCUSSION

The data presented in this paper have confirmed the fact that continued propagation of W.E.E. virus in the mouse brain may yield a viral variant with increased rapidity of action. The greater speed with which such adapted virus killed mice was paralleled by a corresponding increase in its rate of multiplication in the brain. This was associated with a shortened initial latent period during which there was no measurable increase in viral titer.

In mouse brains infected with W.E.E. virus, the titer of recoverable virus was 90 to 96.5 per cent lower than the theoretical yield, and it was at this reduced level that the virus maintained itself for about 3 hours in the case of the fast R.I. strain and for about 5 hours in that of the slow Kelser strain. The initial drop in detectable virus may have been due simply to leakage into other tissues, or it may have been caused by adsorption of a proportion of the inoculum onto host cells with resulting loss of infectivity. The observation that losses of similar magnitude followed the intracerebral inoculation of bacteriophage does not necessarily rule out the latter possibility. With the nature of this initial loss unexplained, it remains doubtful whether the latent period represented a temporary equilibrium between rate of disappearance from the brain and rate of virus increase, or whether it was due to a latent phase in the intracellular growth cycle comparable to the "constant periods" described for bacteriophage (8) and influenza viruses (9). A more conclusive interpretation of the growth experiments is difficult because of the inability to separate unadsorbed or newly liberated virus from infected host cells.

It is interesting, however, that after inoculation of various large amounts of either strain of W.E.E. virus, the rates of multiplication tended to converge, while after inoculation of the R.I. strain in amounts closer to the mini-

mal lethal dose they tended to parallel each other. This latter finding suggests that of the small amount of virus all was utilized in infecting host cells. Convergence in the higher range may indicate that an increasingly high proportion of the seed virus was in excess of the amount immediately utilized to initiate infection. It will be shown in the following paper (2) that this postulated excess may have an important function as free antigen in the mechanism of immunity in vaccinated animals.

The growth experiments here reported, while of some obvious interest in relation to the broader problems of virus-host relationship and viral adaptation, have their chief significance in connection with the difference in response of immunized animals to intracerebral challenge doses of the "fast" and the "slow" variants of W.E.E. virus. This difference was so striking that one may suspect serological heterogeneity. That minor serological changes may occur upon continued propagation of viruses in certain hosts has been suggested by studies on influenza virus (10). Careful investigation, however, has failed previously (1) and again in the present work to reveal detectable serological changes resulting from adaptation of W.E.E. virus to the mouse brain. The mechanism which enables vaccinated animals to survive infection with a "slow" strain but not with a "fast" derivative from it will be described in the following paper (2).

The different response of vaccinated animals to 2 serologically indistinguishable variants of the same virus may have some practical significance. It is conceivable that active immunity tests with variants of other viruses having similar differences in growth rates may have created the impression that they represented strains of different immunological types. This possibility should be considered especially in the case of poliomyelitis virus where immunological differentiation of strains has often been based on active immunity rather than on neutralization tests.

Similarly, certain standard potency tests to which vaccines prepared for medical and veterinary use are subjected involve intracerebral challenge inoculations in vaccinated mice. Habel and Wright (11) have recently recognized that various strains of rabies virus when used for challenge in such tests may cause wide variations in the results obtained, and on this basis they have recommended the use of aliquots of a single sample of standard challenge virus in all laboratories.

The results and conclusions presented in this and in the following paper (2) for W.E.E. virus may apply equally to similar variations encountered with other viruses.

SUMMARY

Continued serial brain-to-brain passage of strains of W.E.E. virus in mice has yielded variants which kill mice with increased rapidity. Their rate of

multiplication in the mouse brain has been found to be correspondingly increased.

At 1 hour after intracerebral inoculation of various amounts of W.E.E. virus, only 3.5 to 10 per cent of the expected amount of virus was recovered from the infected brains.

In infected mouse brains, the period of active viral multiplication was preceded by a latent phase which lasted a considerably shorter time in the case of a "fast" than in that of a "slow" variant.

In brains inoculated with various amounts in excess of minimal lethal doses the rates of multiplication tended to converge with the result that the maximum titer was reached after about the same period of time. After inoculation of smaller amounts, the rates of viral multiplication tended to parallel each other.

Vaccinated mice may be fully resistant to maximal intracerebral doses of a slowly multiplying strain while they are not at all or only partly protected against a rapidly multiplying one derived from it. This difference is demonstrable even though fast and slow variants are, as far as can be tested, serologically identical. The difference in response may be masked if animals are immunized with relatively large doses of vaccine.

The bearing of these findings on certain practical problems has been pointed out.

BIBLIOGRAPHY

1. Olitsky, P. K., Morgan, I. M., and Schlesinger, R. W., *Proc. Soc. Exp. Biol. and Med.*, 1945, **59**, 93.
2. Schlesinger, R. W., *J. Exp. Med.*, 1949, **89**, 507.
3. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.
4. Lauffer, M. A., and Miller, G. L., *J. Exp. Med.*, 1944, **79**, 197.
5. Horsfall, F. L., Jr., and Curnen, E. C., *J. Exp. Med.*, 1946, **83**, 25.
6. Sabin, A. B., Duffy, C. E., Warren, J., Ward, R., Peck, J. L., and Ruchman, I., *J. Am. Med. Assn.*, 1943, **122**, 477.
7. Ruchman, I., *J. Immunol.*, 1946, **53**, 51.
8. Ellis, E. L., and Delbrück, M., *J. Gen. Physiol.*, 1939, **22**, 365.
9. Henle, W., Henle, G., and Rosenberg, E. B., *J. Exp. Med.*, 1947, **86**, 423.
10. Francis, T., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 143.
11. Habel, K., and Wright, J. T., *Pub. Health Rep., U.S.P.H.S.*, 1948, **63**, 44.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, June 1, 1949,
Vol. 89, No. 6, pp. 583–596]

PERIPHERAL VASCULAR REACTIONS IN ANAPHYLAXIS OF THE MOUSE

BY PHILIP D. McMASTER, M.D., AND HEINZ KRUSE

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

PLATE 29

(Received for publication, January 22, 1949)

In the course of studies on the site of antibody formation it was found that mice sensitized to horse serum showed extraordinary vascular reactions in the ears when reinjected with the same serum at an appropriate interval. The phenomena, observed under the microscope, appeared not only in animals showing anaphylactic shock but also in many which presented no other apparent signs of anaphylaxis. That is to say the vascular changes in the ear seemed to constitute a sign of anaphylactic sensitivity far more delicate than the production of anaphylactic shock itself. It seemed likely that these reactions could be used for the study of the mechanisms of local hypersensitivity. Further, it seemed probable that by means of these reactions the mouse could be used as a convenient laboratory test animal to take the place of larger or more costly animals for various immunological studies.

Because of erroneous statements in the older literature (1-7) it is still believed, even by many immunologists and allergists, that the mouse is not susceptible to anaphylactic shock, and the possibility of using the vascular changes in its ears for immunological research has, of course, not been considered. Under these circumstances it seemed wise to make a study of the characteristics of the peripheral vascular responses in mice showing anaphylactic shock in all degrees of intensity, not only to learn more about them but to determine whether they are a true part of the anaphylactic reaction—and hence an indication of the animal's sensitivity—or whether they are merely secondary to blood pressure changes or nerve stimuli.

The present paper describes the findings of such a study carried out in mice sensitized to various sera and reinjected later with the same material. The vascular changes will be fully described since they have brought out some new findings of physiological interest quite apart from their immunological implications.

Although a number of workers (8–17) have observed anaphylactic shock in the mouse their studies have not been aimed at an understanding of the circirculatory changes taking place in this animal. On the contrary little is known about this matter. Fortunately for us methods for observing the most minute as well as the larger blood vessels in the ears (18) or claws (19) of mice, and for measuring their blood pressure during and after anaphylactic shock were already at hand in this laboratory (19).

Previous Work.—In spite of the fact that several authors (1–7) reported between 1908 and 1910 that mice are refractory to anaphylactic shock, papers by Braun (8, 9) and Schultz and Jordan (10) appeared within about a year of each other, proving the contrary. Shortly thereafter Ritz (11) and von Sarnowski (12) also produced anaphylactic shock in mice, and in 1926 Schiemann and Meyer (13) obtained both active and passive anaphylaxis. In 1937 Bourdon (14) reported active sensitization of white mice and recently Weiser, Golub, and Hamre (15) have restudied the subject and discussed the previous findings. The mouse has also been used by Mayer and Brousseau (16) and also by Perry and Darsie (17) to gage the activity of antihistaminic drugs and to study histamine shock.

The Induction of Anaphylactic Shock in Mice

In the present experiments trial and error showed that mice could be rendered anaphylactically sensitive, as a rule, by injecting 0.03 cc. of horse, pig, or rabbit serum into the peritoneal cavity twice, at an interval of 48 hours. After an appropriate period shock was induced by injecting into a tail vein 0.05 to 0.15 cc. (usually 0.1 cc.) of the sensitizing serum, per 30 gm. of body weight. Optimum results were obtained when the mice were shocked 16 to 35 days after the first sensitizing injection. Under these circumstances about three-fourths of the mice showed the pronounced shock symptoms that will be detailed below. About 20 per cent of them died 20 minutes to 2½ hours after the injection, and another 10 to 20 per cent died during the next 12 hours. The reactions were less regular and less severe when shorter or longer time intervals elapsed between the sensitizing and shocking injections, but shock was obtained as early as the 9th day and as late as 6 months after the first sensitization. Longer intervals have not been tried. Usually, however, shocking injections made at an interval of more than 7 weeks gave irregular findings.

Types of Experiments

Normal white mice of the Rockefeller Institute strain were employed throughout. Both unanesthetized and anesthetized animals were shocked in order to correlate the time relationship between the physiological changes, which could be best observed in the latter, and the behavior phenomena displayed by the former. Single intraperitoneal injections of pentobarbital, 0.5 cc. of a 1 per cent solution per 30 gm. of body weight, yielded suitable anesthesia with which to observe the circulatory changes in the ears or claws and to measure the blood pressure changes in the carotid and femoral arteries. These observations were made before, during, and after shock, by methods to be outlined below. The blood pressure measurements could, of course, be carried out only in the anesthetized animals, but it should be stressed here that the vascular changes, observed to the best advantage in the anesthetized mice as described further on, were also observed, with some difficulty, taking place in unanesthetized ones, at the same times after the injection of antigen and with the same intensity as in the anesthetized animals. It follows that anesthesia was not responsible for the findings.

Signs of Anaphylactic Shock in Unanesthetized Mice

The earlier workers are agreed (8–17) that anaphylactic shock in the mouse is a less stormy event than in the guinea pig or rabbit. For the first 5 or 10 minutes unanesthetized mice show merely agitation or hyperexcitability (15). Later they scratch themselves (10), the hair becomes ruffled, respiration is difficult, and some throw themselves here and there (12) or have brief convulsions with long, quiet periods between (15). Cyanosis in the ears and feet has been noticed (12, 15), but a prior blanching has not been mentioned although, as reported below, it appears regularly soon after the shocking injection is given. There is often a frog-like posture with the hind legs extended behind the body (15). Death does not come on

rapidly as in shocked guinea pigs and rabbits, but after 15 to 60 minutes (15), or it may occur after many hours.

We have found the picture of shock induced by horse, pig, or rabbit serum in unanesthetized mice substantially like that described above, with one great exception. Many of the animals showed either no signs when observed in the gross, or at most only agitation and restlessness; but when their ears were examined at low magnification after they had been placed in a holder, one observed striking vascular reactions like those now to be described as occurring in perfectly quiet anesthetized mice. They differed only in degree, being less severe, than those seen in mice with outspoken anaphylactic shock.

PERIPHERAL CIRCULATORY CHANGES TAKING PLACE IN ANESTHETIZED MICE WITH ANAPHYLAXIS

In contrast to the delay in the appearance of the manifestations of anaphylaxis, the microscope revealed profound physiological changes in the blood vessels of the ears and claws taking place promptly. They appeared even during the injection of antigen, and long before unanesthetized animals showed any signs of distress.

Changes in the Circulation of the Ears.—In more than 300 experiments anesthetized normal or sensitized mice, approximating 30 gm. in weight, were placed prone in plastaline moulds with their ears spread out upon white porcelain placques, in a manner previously described (18–20). By these means even the most minute, as well as the larger, vessels were observed in the intact ears under the microscope. Next, one worker injected 0.05 to 0.15 cc. of serum into a tail vein while another observed the ear vessels during the injection and for various periods thereafter. The injection required 30 seconds to 1 minute.

At any time from 28 seconds to 7 minutes after beginning the injection, but usually between the 50th and 90th seconds, vascular reactions began, if the animals were sensitive.

Changes in the Arteries.—Usually there appeared first one or more brief, partial contractions extending all along the arteries of the mid and peripheral portions of the ears. In a second or two the vessels returned to their initial calibers. Momentarily the circulation increased in speed, but then, after a few seconds, it became much slower than it had been before the injection. Next, sharply localized contractions appeared in many arteries. Some promptly relaxed, but others persisted. In highly sensitive animals the localized spasms increased in number and intensity, until, within a minute or two, all arterial vessels were completely constricted and had disappeared from view. In less sensitive mice only some of the arteries showed a complete obliterative spasm while in the others the local constrictions remained, trapping blood in the vascular segments lying between them. In poorly sensitized mice only a few localized spasms were seen. More will be said of these differences below.

In those instances in which vascular spasm did not occur for a minute or two, the slowing of the circulation was the most prominent feature. Cells, moving in clumps, separated by plasma could be seen, as though they had become sticky and adherent. In rare instances flow ceased in ear arteries which were still patent, showing that spasm must have occurred in larger, more centrally situated arteries than those of the ear. In the vessels that had become completely occluded blood flow stopped of course, but in the vessels in which constriction was partial the flow merely remained slow.

Changes in the Veins.—The veins also contracted. Usually sharply localized, ring-like constructions occurred, obliterating the lumina of the vessels for only a small fraction of a

millimeter while the greater part of the veins remained widely open. As in the arteries, many of the constricted portions relaxed after a few seconds while new spasms appeared elsewhere, or, the original constrictions remained while the newer ones occurred, until the veins became segmented in appearance, or completely constricted. Occasionally widespread constriction of entire vessels took place at once.

The intensity of the reactions varied much from one batch of mice to another. By and large about 20 per cent of the sensitized animals showed almost complete obliterative spasm of all the ear arteries and veins for several minutes. In another 30 per cent spasm of the vessels was severe enough to produce complete obliteration of one or more arteries and veins in each ear, with partial or local obliteration of segments of many of the remaining vessels. About 17 per cent of the animals showed marked constrictions in arteries or veins but no obliterative spasm. About 10 per cent showed no constrictions, but instead stoppage or slowing of the circulation. The remainder, about 23 per cent, gave no visible reactions at all. No spasm or constriction of vessels was ever observed in the scores of control, unsensitized mice, injected with the same sera.

In severe shock, when complete obliterative spasm occurred in both veins and arteries, almost no blood remained in the blanched ears. In mild shock general obliteration of the arteries and veins was absent, and the local constrictions trapped the blood in the unconstricted segments, as has been mentioned above.

It is a matter of much interest that, in occasional instances the veins constricted before the arteries and in a few instances of mild shock spasms appeared in the veins while the arteries never showed constrictions at any time. The findings show that the venous constrictions were not brought about by a lack of blood in the vessels. Indeed, as will be seen below, all the constrictive changes took place while the carotid blood pressure stood either at the initial normal evel, or 10 to 40 mm. of mercury above it.

The Appearance of the Capillary Bed.—The appearance of the capillary bed differed much from animal to animal. To understand the differences it may be best, for descriptive purposes, to consider the vascular changes just described as falling into three general types; one in which arterial spasm set in before venous constriction; another, the commonest, in which arterial and venous spasm were synchronous; and finally, a relatively infrequent type characterized chiefly by venous spasm. Of course every possible combination of these types occurred, but as will be seen from what follows the happenings in the capillary bed were conditioned by the state of affairs in the larger vessels.

In most of the experiments in which there was obliterative spasm of the ear vessels the capillary bed blanched and the capillaries became invisible. This phenomenon occurred both in the instances in which arterial spasm preceded venous constriction and in those in which arterial and venous spasm occurred simultaneously. The capillary bed seemed to have emptied into the veins. An observer looking at the ear for the first time during this stage of shock might have readily assumed that an obliterative capillary contraction had occurred. However, this was not the case. In many experiments, one or two true capillaries (as defined by Chambers and his coworkers (20–23) were watched in one ear, at magnifications ranging from ×450 to ×900, while the changes in the larger vessels of the other ear were followed

through a low power (×80) microscope by another observer. When the capillary bed became blanched, as seen by the latter, the watcher at high power also lost sight of the capillaries he had been observing. Nevertheless, as he continued to search the spot where they had been visible, single blood cells suddenly appeared from time to time, passing rapidly through the invisible capillaries, which were obviously still patent but full of fluid. Presumably pressure from incompletely obstructed arteries skimmed off plasma which maintained a current flowing through the capillaries in which scattered blood cells moved on their way toward the veins.

When constriction of the veins occurred before that of the arteries, the true capillaries became choked with closely packed red blood cells. An observer seeing the ear at this stage for the first time might have remarked upon "capillary dilatation." However this is not the case; the capillary bed was simply pumped full with packed red cells.

It is clear from scores of such observations that the true capillaries of the mouse's ear are passive in anaphylactic shock; what happens in the capillary bed is determined by the site of spasm or constriction in the larger vessels. There is no contraction of the true capillaries, an observation which is in agreement with the recent findings of a number of workers studying other forms of shock (20–23).

The Recovery of the Circulation in the Ears.—In all mice that survived more than a few minutes, even in those that died after several hours and in those that, while surviving, yet showed complete obliterative spasm, recovery of the circulation in the ears began about 5½ to 20 minutes after beginning the serum injection. The first movement of blood took place either in the veins or in the arteries. In instances in which both the capillary and venous beds were blanched, the earliest movement of blood began in the arterioles and was followed at once by a prompt surge of the cells into the already patent capillaries. In a surprisingly short time all the vessels in the ear became filled with blood and widely distended. In the instances in which the capillary bed was already filled with cells, the direct arteriolar-venular channels (the A-V bridges of Chambers and Zweifach (20–23) opened first and blood passed through them into the venules, regardless of whether the latter were filled or empty. As result, in all instances the venules became pumped full of blood cells as recovery progressed, and true capillaries leading to the venules often became even more distended by a reverse flow. Very rarely one saw a plug of well packed cells forcing its way through a minute vessel. The ears soon appeared in the gross as though in a state of flaming hyperemia, but under the microscope one could plainly see that the movement of blood was excessively slow. As will be seen below the systemic blood pressure during this stage was low.

Figs. 1 to 6 illustrate, at low magnification (× 25), the changes that occurred in an ear during a moderately severe shock. The first photograph shows the ear before the serum injection, the second at one minute and a half after beginning it. Local obliterative spasms had occurred in practically all the vessels, trapping blood in them between the constricted segments. Fig. 3 shows the ear as it appeared 5 minutes after the injection. The arteries, together with many veins, had disappeared, but trapped blood can be seen in many other veins In this instance complete obliterative spasm did not occur, but the ear, save for the trapped blood, was blanched and white and the capillary bed was empty. Fig. 4 shows early recovery, 12½ minutes after the beginning of the injection. Veins here and there were filling with blood, but the arteries were scarcely

visible except under a higher power. Then one saw a hesitant, intermittent *trickle of blood passing through them. Within 4 minutes more, however, (16½ minutes after the injection) all vessels in the ear had filled with blood (Fig. 5). The veins, distended almost to their initial calibers, were choked with closely packed, scarcely moving, blood cells. The capillary bed, too, had filled,* but the arteries were still narrow and thread-like. Finally, half an hour after the injection (Fig. 6) the distended vessels gave to the ear the appearance of an intense hyperemia; but actually, for reasons to appear below, blood was scarcely moving through the organ.

In anticipation of findings to be given almost immediately below, it can be said that at the time that Figs. 2, 3, and 4 were taken, the carotid blood pressure was far above normal—and when the last two photographs were taken, it was far below normal.

THE BLOOD PRESSURE CHANGES AND THEIR RELATIONSHIP TO THE OTHER ANAPHYLACTIC PHENOMENA

It is generally believed that the clinical picture of anaphylactic shock results from some effect of the antigen-antibody reaction which leads, directly or indirectly, to constriction of smooth muscle in blood vessels, bronchioles, and other structures. A fall in blood pressure as a regular accompaniment of anaphylactic shock has been found in all animals in which suitable studies have been made. This has been ascribed by many to interference with the return of blood to the heart because of the constrictions of large veins in the abdomen or chest, a supposition substantiated by many *in vitro* experiments carried out with the Schultz-Dale technique and by the pathological findings in the lungs, liver, and other viscera in shocked animals. However in the later phases of anaphylactic shock there exists, along with low blood pressure, a profound vasodilatation. One would expect to find a compensatory vasoconstriction. Is the vasodilatation, therefore, an independent reaction, the result of the antigen-antibody reaction, or perhaps caused by nerve stimuli?

It is not known with certainty what rôle is played by the nervous system in the intact animal undergoing anaphylactic shock, nor what is the relationship between the vascular spasms, or subsequent dilatations, and the blood pressure changes. It seemed probable that the techniques for observing peripheral vascular changes here described, when combined with blood pressure measurement, might be used to answer some of these questions. We first determined what blood pressure changes, if any, occur in mice since this had not been previously studied. Next, after profound changes had been found, the time relationships between them and the onset of vasopasm and later dilatation were studied to learn which came first; that is to say, whether the vascular changes took place in compensation for the blood pressure changes or *vice versa,* whether they were occasioned by nervous stimuli, or whether they were independent reactions apparently brought about by the antigen-antibody reactions. The work has

shown that the vascular responses are independent of the blood pressure changes and furthermore are not determined by nervous stimuli.

Methods.—A previous paper from this laboratory (19) has described two methods for measuring blood pressure in the same mouse, one directly, by cannulation of the carotid artery, the other indirectly, by transillumination of the claws and observation of the blood flow in the claw bed, following inflation of a sphygmomanometer cuff placed about the thigh. Simultaneous measurements by both methods showed excellent agreement.

For the present work, mice, anesthetized with nembutal (19), were placed on their backs in the apparatus for measuring blood pressure (19), while their heads were supported in such a manner that the ears, with their dorsal surfaces facing downwards, spread out very lightly and without tension on glass slides. A few centimeters below the ears a mirror was fixed, and a strong cooled light, directed at an angle from below upwards, illuminated the dorsal surfaces of these organs, and allowed the brilliant reflexion in the mirror to be magnified by a binocular microscope. By these means the state of the blood vessels in the ear could be observed while blood pressure determinations were made at frequent intervals.

Thirty-two experiments were done. Eight normal mice were injected intravenously with 0.2 cc. of horse serum per 30 gm. of body weight. A second group of 16, sensitized to horse serum, were injected in the same way with similar amounts of the same serum. Of these, 11 showed spasms in the vessels of the ears. The remaining 8, sensitized to horse serum, were given intravenous injections of similar amounts of rabbit serum; that is to say, material containing proteins to which they had not been sensitized. In all, the systolic blood pressure was measured by both of the methods already mentioned (19) before, during, and after the serum injections, each of which required approximately 1 minute to complete.

Findings.—As already reported (19) the systolic blood pressure in the normal mouse anesthetized with nembutal or luminal, varies between 118 and 60 mm. of mercury depending largely upon the depth of the anesthesia. By the time that surgical anesthesia has been induced by the intraperitoneal injection of these anesthetics the systolic blood pressure is usually low, 60 to 90 mm. of mercury, and it may remain low for 15 to 45 minutes longer. As the anesthesia becomes lighter the pressure rises. Consequently in all the experiments to be considered here the pressure measurements were made at approximately the same time (45 minutes) after injecting the anesthetic. Only figures for the systolic pressure will be given hereafter.

Prior to the injections the systolic carotid blood pressure of all the mice ranged between 85 and 108 mm. of mercury. In some of them pricking the skin of the tail to inject the vein lowered the pressure by 5 to 8 mm. of mercury, but repeated prickings caused a rise of about 5 mm. Invariably the blood pressure was allowed to return to the previous level before the serum injections were begun. About 30 seconds after beginning the injections, when approximately half the dose had been given, the blood pressure began to rise by about 10 mm. of mercury. The rise continued to its maximum, usually 15 to 25 mm. above the preinjection level, in 1 to 3 minutes after the injection was completed. Occasionally the pressure rose by 30 to 40 mm. of mercury.

In the 8 normal mice, and in the 8 horse serum-sensitized ones injected with rabbit serum, there followed a gradual fall to the original pressure in the following 15 to 30 minutes. The blood vessels of the ears dilated slightly in some instances or remained unchanged while the circulation rate increased and the pulsation in the manometer in the carotid artery became greater.

Quite different were the findings in the 11 horse serum-sensitized mice which showed anaphylactic shock. In these the carotid blood pressure rose, as in the others, but at periods varying between 50 seconds and 3 minutes after the beginning of the injection, and either while the carotid blood pressure was still rising or while it remained at or near its maximum

height, spasm and constriction of the blood vessels of the ears took place. In several instances there was complete obliterative spasm of all the ear vessels and the organ was quite bloodless while the carotid pressure stood at levels 20 to 40 mm. of mercury higher than before the injection.

About 4 to 5 minutes after the beginning of the injection the blood pressure of the 11 mice that showed spasm of the ears began to fall while the spasm yet endured. Within 8 to 10 minutes the pressure usually reached the preinjection level, sooner than in the unsensitized mice or those receiving non-antigenic serum. One or 2 minutes later, when the pressure had reached a level about 20 mm. of mercury lower than the preinjection level, some of the ear vessels began to dilate and restoration of the circulation began. Thereafter the carotid blood pressure fell very rapidly, to 35 to 40 mm. of mercury, or even to 20 mm. or lower. The arteries and veins of the ears became more dilated, and the slow, stately flow of blood, already described, appeared in all of them. If death did not occur the blood pressure remained at levels between 20 to 45 mm. of mercury for an hour. Thereafter, further measurements by the direct method were abandoned.

Later Blood Pressure Changes. Studies of the Circulation in the Claws.—By the method for transillumination of the claws, already described (19), one observer watched the circulation in a claw bed of a hind toe, while another watched the mirror image of the ear vessels and noted the carotid blood pressure. The normal mice, injected with horse serum, and the horse serum–sensitized ones given rabbit serum, showed the usual rise in blood pressure, when the latter was measured in the leg, and also an increased circulation in the vessels of the claws. By contrast the circulation of the sensitized-shocked mice ceased at the same time that spasms began in the ear vessels, while the carotid blood pressure stood at the same high levels observed in the other animals which received serum injections, but in which there had been no blood vessel changes. The stoppage took place too rapidly to permit one to make pressure measurements by the indirect method. The blood cells in the claw bed remained immovable within the capillaries, which showed neither constrictions nor dilatations. When the circulation began again in the ears blood flow also reappeared in the claws of about half of the animals, indicating that in these instances spasm of the peripheral vessels in the ears and legs had relaxed simultaneously. In the remaining mice circulation in the claws reappeared later than in the ears, by periods ranging from 1 minute to 2 hours.

Since the blood flow in the claws ceased at the same time that vascular spasm occurred in the ears, the indirect method for measuring blood pressure in the legs was found useless during the acute stage of shock. However, animals with cannulated carotid arteries can be employed for limited periods only, and it was found possible to use the indirect method to advantage to study pressure changes occurring in the long period of recovery from shock. In 20 additional experiments blood pressure readings were made in the legs before shock was induced and again after shock, as soon as transillumination of the claws showed that blood flow had been resumed in the legs. The readings were repeated at intervals during the long period of recovery, or in the hours preceding delayed death, as the case might be.

When fatal shock occurred the blood pressure fell progressively to levels below 20 mm. of mercury and the animals died, either at once or within 1 to 3 hours. In the majority of survivors the pressure did not fall below 35 to 45 mm. of mercury, but even in these it required several hours to return to normal. The pressure of 4 of the 20 animals hovered between 18 and 30 mm. of mercury for 3 or 4 hours, the prostrated animals requiring no anesthetic or restraint while making the pressure measurements. Three of these died, but in the animal that survived the pressure began to rise slowly after the 3rd hour.

The findings show conclusively that the vasoconstriction in the ears and legs of the sensitized-shocked mice was not compensatory to a fall in blood pressure, since all the animals showed normal or elevated blood pressures at the time the

constrictions began. As shown by the experiments upon normal mice, part of the rise in blood pressure was due to the increase in blood volume occasioned by the injection of a relatively large amount of serum—0.1 cc. is equivalent to about 4 per cent of the blood volume of a 30 gm. mouse. The remainder of the rise in the shocked animals was conditioned, no doubt, by the closing off of many areas of the peripheral vascular bed by spasm of the arteries.

It is of interest that the carotid blood pressure began to fall while peripheral vasular spasm was still active, at least in the vessels of the ears. After the pressure had fallen to levels a little below the normal, vasodilatation took place, a reaction that can in no way be construed as compensatory in nature. It seems reasonable to assume that the vasodilatation was a primary reaction, and that it produced the fall in blood pressure. A vasodilatation occurring in the viscera or muscles could bring about the fall in carotid blood pressure even while the ear vessels remained in a constricted state. Work to be reported later has shown that such a vasodilatation does take place in the mouse.

THE PATTERN OF THE VASCULAR REACTIONS IS COMPLETELY INDEPENDENT OF THE BLOOD PRESSURE CHANGES AND OF NERVOUS CONTROL

Further experiments have shown more clearly that both the vasospasm and the subsequent vasodilatation of the ear vessels are wholly independent of the state of the blood pressure in the large arteries and that they are not brought about by nervous control.

It is generally believed that nerve stimuli are not of fundamental importance in the anaphylactic reaction. Seastone and Rosenblueth (24) have shown that the denervated nictating membrane of the cat contracts in anaphylactic shock. Lissak and Hodes (25) showed that anaphylactic shock of cats was similar, whether or not the sympathetic nervous system had been ablated, and Lissak and Kokas (26) found atropine to be without effect upon the progress of anaphylactic shock in dogs. These experiments, however, have ruled out special systems and specific mechanisms, not the nervous system as a whole, and additional evidence for or against the participation of the latter in anaphylactic shock is much to be desired. The techniques employed in the present work seemed to offer an excellent opportunity to throw light upon these questions.

Mice, sensitized and anesthetized as usual, were placed in plastaline moulds with only one ear lying on a porcelain placque. The other ear was spread horizontally over a smooth cork which had been selected and carved for each experiment until it fitted perfectly into the auditory meatus of the mouse to be studied. The corks were supported in the plastaline moulds, and the pinnae lay smoothly over them with result that the blood flow in the slightly curved upper surfaces of the ears could be observed through the binocular microscope in the usual manner. A rubber band, 3 mm. wide, attached to a scale pan, was slipped over the ear supported by the cork and adjusted close to the ear base. While observing the circulation in the vessels, weights were added to the scale pan until the arterial circulation and all movement of blood in the ear ceased. Usually about 22 to 28 gm., inclusive of the weight pan, sufficed.

The usual shocking dose of serum was next given to the animal, and the vascular reactions were watched in both the obstructed and unobstructed ears.

The findings from 12 experiments of this sort have been summarized in Table I, with the individual instances arranged from the top downwards in relation to the speed at which spasm appeared in the unobstructed ear following the injection of the antigen. Complete obliterative spasm of the ear vessels occurred in 5 of the 12 mice (4+ in column *b*). In two other animals approximately three-quarters of the vessels went into spasm (3+), in 3 others half of the vessels (2+) were constricted, more or less, and in one, less than one-quarter (1+)

TABLE I

The Speed of Onset and the Intensity of Anaphylactic Vascular Reactions in Ears with Free Circulation and in Ears Subjected to Temporary Circulatory Obstruction during the First Phase of Anaphylactic Shock

In ears with free circulation			In ears subjected to temporary circulatory obstruction				
(*a*)	(*b*)	(*c*)	(*d*)	(*e*)	(*f*)	(*g*)	(*h*)
Time to onset of vascular reactions*	Degree of spasm‡	Period of recovery§	Interval between recovery and release of block	Total period of block‖	Time to onset of spasm¶	Degree of spasm‡	Period of recovery**
min.		*min.*	*min.*	*min.*	*min.*		*min.*
0½	4+	10¾	6	18	0¾	4+	9¼
0¾	2+	11½	15	28	6½	2+	5½
0¾	4+	16	60	77	4½	4+	12
1⅓	3+	13	15	30	1	3+	4¾
1⅓	4+	10¾	24	36	0⅔	3+	14
1½	3+	16	8	25	8	2+	16
1½	4+	17½	25	40	0½	2+	14½
1¾	2+	7	19½	28	4	2+	12
2	2+	5½	5	12½	2	2+	5⅔
2	4+	10	3	14½	3¼	2+	14
3¼	1+	8	7½	16½	8	1+	17
One other animal showed no reaction in either ear.							

* After the beginning of the injection of antigenic serum.

‡ See text.

§ Time after the beginning of the injection, at which blood flow began again.

‖ These figures represent the total period of circulatory block from the time that the rubber band and the weights were placed over the ear until they were removed.

¶ After the return of blood to the ear.

** The time after the return of blood to the ear.

In one mouse there was no reaction. No correlation appeared between the speed of onset of spasm and the intensity of the reaction. In all these animals the spasm of the vessels relaxed in 5½ to 17½ minutes after the injection began and recovery of the blood flow took place (Column *c*).

In no instances were any changes noted in the caliber of the vessels of the obstructed ears. Had nervous impulses initiated the constrictions in the unobstructed ears there should have been spasm of vessels on the obstructed side, for the periods of circulatory stoppage at the times that the vascular spasms

occurred in the unobstructed ears were not long enough—less than 4 minutes—to block nerve impulses, nor was the mild pressure of the rubber band sufficient to do so.

Final proof that the vascular reactions were completely independent of the blood pressure changes, indeed that they probably provoked the latter, was obtained in the following manner.

At intervals ranging from 3 minutes to 1¼ hours after recovery of the blood flow in the unobstructed ears the rubber bands were removed from the obstructed ones. At once blood flow commenced and in every instance except No. 12 (Table I) in which there was no reaction in the unobstructed ear, spasm and constriction took place in the experimental ear after the remainder of the animal's body had passed through its shock reaction. As the table shows (column e) the onset of spasm after the first influx of blood in the experimental ear was sometimes quicker, sometimes slower than in the other ear, and the time required for recovery differed somewhat. The intensity of the spasm in the two ears of the same animal also differed slightly but by and large the reactions were like those observed in the unobstructed ear, during the initial shock. It was obviously a local shock reaction, a replica of the preceding one in the other ear.

It is important to note that, after release of the circulatory obstruction, vasoconstriction took place while the animals' blood pressure must have been very low, which was always the case when measurements were made in the mice during the recovery phase. By contrast, when the constrictions took place in the unobstructed ears the pressure must have been higher than normal. The inference is clear, that the pressure had no effect upon the occurrence of the vasoconstrictions.

DISCUSSION

The chief finding of the present work would seem to be the occurence of vasospasm and arrest of the circulation in the ears and feet of sensitized mice undergoing anaphylaxis. Such changes occurred not only while the blood pressure in the larger arteries was normal or slightly elevated, but at a time when unanesthetized animals, examined in the gross, showed either no symptoms whatever or at most only mild agitation and restlessness. The microscope revealed, both in unanesthetized and anesthetized animals, profound peripheral vascular effects imperceptible to the unaided eye. In the majority of cases the alteration was not fatal, and since it was to be perceived only by special methods, it can be termed occult anaphylaxis.

As already brought out, the constrictions and spasms of the ear vessels occurred only in sensitized mice, not in those previously normal. These reactions were primary phenomena in their own right and not dependent upon the changes in blood pressure or nervous stimuli. In mice poorly sensitized and showing little or no true shock at any time they were milder than in those

that passed later into severe or fatal shock, but the differences were of degree only, not of character. It is well known that sensitized or immunized mice yield notably poor skin reactions. This inference seems warranted, that the behavior of the ear vessels may be of service in the laboratory to detect slight degrees of anaphylactic sensitivity which might be missed by other means of investigation.

None of the earlier workers has studied the small vessels of the mouse during anaphylaxis. There has been no discussion of the reactions of minute peripheral vessels in sensitized animals not sufficiently hypersensitive to undergo actual anaphylactic shock. In two laboratories, however, constrictions and spasms of the vessels of the rabbit's ear have been observed during severe shock. Abell and Schenk (27) described them occurring within a Clark chamber in the animal's ear, and Bally (28–30) noted their appearance in the ears of rabbits during histamine, peptone, and anaphylactic shock. In this last and in histamine shock severe constrictions of the large arteries were seen early, about a minute and a half after the shocking injection, and after about 2 minutes, constriction of all the vessels took place. The blood pressure remained up until the spasms had reached their maximum, and fell thereafter, just as in our experiments on mice. By contrast, in peptone shock transient dilatation of the ear vessels occurred, followed by a late constriction after the blood pressure had fallen to its lowest point. The reactions witnessed by both groups of workers above mentioned must have been much like those which occur in the mouse.

Blanching in the ears of guinea pigs in anaphylactic shock has scarcely been mentioned in the literature, but a late cyanosis of the organs has been often reported. As is well known the systemic blood pressure first rises and then falls to low levels.

It would seem worthwhile to find out whether the ears of weakly sensitized rabbits and guinea pigs show peripheral vascular reactions in the absence of other signs of shock.

The present observations on mice showing severe shock have brought up some interesting possibilities. As already stated the behavior of the true capillaries of the ears was wholly unforeseen. They did not undergo any active constriction or dilatation, but simply remained patent and full of plasma though empty of red cells, or became crammed and distended with these latter, depending upon the state of affairs in the larger vessels. These findings fall in with those of recent workers (20–23) who have studied the behavior of true capillaries in other forms of shock.

The changes in the veins were pronounced, and the fact that they narrowed before the arteries did, or that in some instances constrictions occurred only in the veins, leads one to wonder whether something of the sort may not take place in skin during the development of wheals and other allergic skin phenomena in man.

It seems probable that the occurrence of occult anaphylaxis in generally sensitized animals showing no other signs of anaphylactic shock, and not locally sensitized, may have implications for the clinic. Allergists have long suspected that something like it happens in the sensitized tissues of man, and its occurence would explain instances of partial collapse, distress, anxiety, and other symptoms for which no cause has as yet been found.

SUMMARY

Pronounced vascular changes occurring in the ears and claws of mice during anaphylactic shock are described. Practically at once after a foreign serum (pig, horse, or rabbit) enters the blood stream of sensitized animals both the arterial and venous vessels undergo marked, local or generalized constriction in the organs mentioned. Usually spasm of the vessel walls occurs simultaneously in the arteries and veins, but it may appear first in the arteries, or occasionally in the veins. When venous spasm precedes arterial spasm, the true capillaries become distended with cells; if the reverse order holds, the ears appear bloodless. There is no active constriction or dilatation of capillaries; the capillary behavior follows passively the changes in the large vessels.

Peripheral vascular spasm occurs while the carotid blood pressure is high, but a few minutes later, while this still holds true, the ear vessels begin to relax and the circulation is resumed. Shortly afterwards the blood pressure falls to levels far below normal, but the vessels remain open.

If the circulation of one ear is obstructed while anaphylactic shock is produced, no vascular spasm occurs in it. Release of the obstruction during the animal's recovery results in belated constriction of the blood vessels of this ear although by now the vessels in the other ear are dilated and the general systolic blood pressure is very low.

The vascular reactions in the ears appear to be uninfluenced by the blood pressure in the large vessels, and they are not a response to nervous stimuli. They are local in origin.

The vascular changes are often not clearly perceptible in the gross but are plainly to be seen under a low power of the microscope. They occur in some sensitized mice exhibiting no manifest signs of shock, differing only in degree from the changes taking place when shock is severe or fatal.

BIBLIOGRAPHY

1. Frey, W., *Arb. Inst. Erforsch. Infektionskrankh. Bern*, 1908, **1,** 66.
2. Trommsdorff, R., *Centr. Bakt., 1. Abt., Orig.*, 1909, **44,** 152.
3. Trommsdorff, R., *Arb. k. Gsndhtsamte*, 1909, **32,** 506.
4. Uhlenhuth, P., and Weidanz, O., Praktische Anleiten zur Ausfuhren des biologischen Eiweissdifferungsverfahrens, Jena, G. Fischer, 1909, 129.
5. Haendl, L., and Steuffenhagen, K., *Centr. Bakt., 2. Abt.*, 1910, suppl. 47, 68.
6. Doerr, R., and Russ, V. K., *Z. Immunitätsforsch.*, 1909, **3,** 181.
7. Galli-Valerio, B., *Z. Immunitätsforsch.*, 1910, **5,** 659.
8. Braun, H., *Münch. med. Woch.*, 1909, **56,** 1880.

9. Braun, H., *Z. Immunitätsforsch.*, 1909, **4**, 590.
10. Schultz, W. H., and Jordan, H. E., *J. Pharmacol. and Exp. Therap.*, 1910–11, **2**, 375.
11. Ritz, H., *Z. Immunitätsforsch.*, 1911, **9**, 321.
12. von Sarnowski, *Z. Immunitätsforsch.*, 1913, **17**, 577.
13. Schiemann, O., and Meyer, H., *Z. Hyg. u. Infectionskrankh.*, 1926, **106**, 607.
14. Bourdon, K. L., *Proc. Soc. Exp. Biol. and Med.*, 1937, **36**, 340.
15. Weiser, R. S., Golub, O. J., and Hamre, D. M., *J. Infect. Dis.*, 1941, **68**, 97.
16. Mayer, R. L., and Brousseau, D., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 187.
17. Perry, S. M., and Darsie, M. L., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 453.
18. McMaster, P. D., and Hudack, S. S., *J. Exp. Med.*, 1932, **55**, 417.
19. McMaster, P. D., *J. Exp. Med.*, 1941, **74**, 29.
20. Chambers, R., *Physiol. Rev.*, 1947, **27**, 436.
21. Chambers, R., and Zweifach, B. W., *Am. J. Anat.*, 1944, **75**, 173.
22. Zweifach, B. W., Lee, R. E., Hyman, C., and Chambers, R., *Ann. Surg.*, 1944, **120**, 232.
23. Zweifach, B. W., Chambers, R., Lee, R. E., and Hyman, C., *Ann. New York Acad. Sc.*, 1948, **49**, 553.
24. Seastone, C. V., and Rosenblueth, A., *J. Immunol.*, 1933, **27**, 57.
25. Lissak, K., and Hodes, B. R., *Am. J. Physiol.*, 1938, **124**, 637.
26. Lissak, K., and Kokas, F., *Arch. exp. Path. u. Pharmakol.*, 1935, **179**, 603.
27. Abell, R. G., and Schenk, H. P., *J. Immunol.*, 1938, **34**, 195.
28. Bally, L. H., *J. Immunol.*, 1929, **17**, 191.
29. Bally, L. H., *J. Immunol.*, 1929, **17**, 207.
30. Bally, L. H., *J. Immunol.*, 1929, **17**, 223.

EXPLANATION OF PLATE 29

The photographs were made by Mr. Joseph B. Haulenbeek.

Figs. 1 to 6. Vascular changes in the ear of a mouse during moderately severe anaphylactic shock. × 25.

Fig. 1. Before the shocking injection.

Fig. 2. 1½ minutes after the beginning of the injection. Local obliterative spasms had trapped blood between the constricted segments.

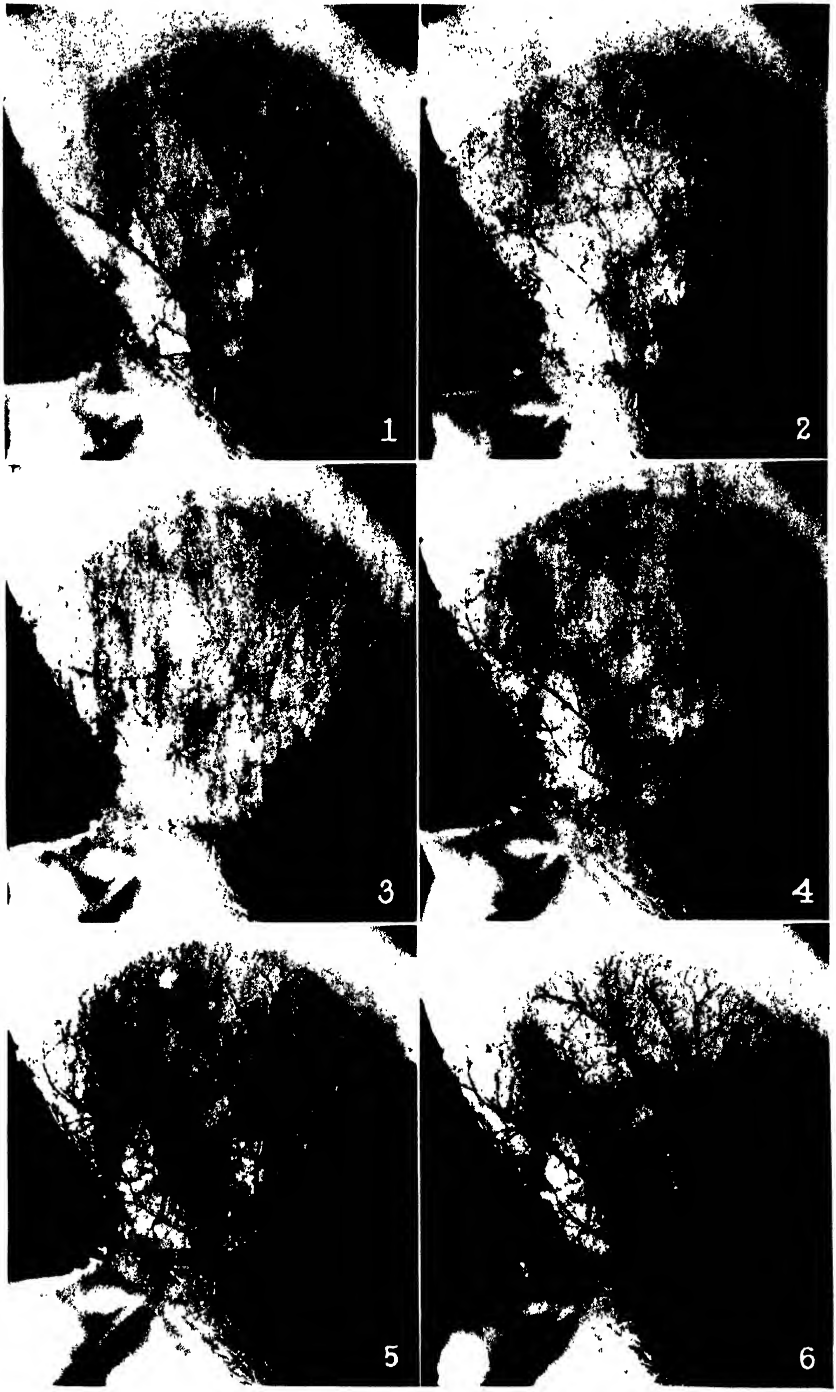
Fig. 3. 5 minutes after the injection. Most of the arteries and many veins had disappeared. In some veins trapped blood can be seen. The ear was blanched, the capillary bed empty.

Fig. 4. Early recovery, 12½ minutes after the injection. The veins were filling with blood which had previously begun to trickle through the scarcely visible arteries.

Fig. 5. 16½ minutes after the injection. All vessels of the ear were filled with slowly moving blood. The arteries were still narrow but the veins had reached their original calibers.

Fig. 6. Half an hour after the injection. The ear appeared intensely hyperemic, but the blood was scarcely moving in the vessels.

When the photographs for Figs. 2, 3, and 4 were taken, the carotid blood pressure must have been above normal—and when the last two photographs were taken it must have been far below normal.


1
2
3
4
5
6

[Reprinted from the ARCHIVES OF BIOCHEMISTRY, June, 1949, Vol. 22, No. 2, pp. 195–203]

THE EFFECT OF LONG-CHAIN CARBON COMPOUNDS, PARTICULARLY HYDROCARBONS, ON THE METABOLISM OF TUBERCLE BACILLI

BY ARTHUR KENNETH SAZ*

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, January 5, 1949)

INTRODUCTION

The utilization of hydrocarbons as energy source by numerous genera of bacteria is a well established fact. The literature on the subject has been adequately reviewed by ZoBell (1). The *Mycobacteria* have been among those organisms particularly implicated in the bacterial degradation of hydrocarbons. Söhngen (2), as early as 1913, showed that at least 6 species of soil *Mycobacteria* were able to attack paraffin wax as well as other hydrocarbons. Others (3, 4, 5, 6) have reported that nonpathogenic *Mycobacteria* from various sources are able to assimilate and decompose various pure or impure hydrocarbons, aromatic as well as aliphatic. Haas (7) reported that *Mycobacterium leprae*, *M. phlei* and *M. smegmatis* oxidized both light and heavy petroleum oils and paraffin wax, but his attempts to grow mammalian tubercle bacilli in hydrocarbon media failed, although an avian strain grew slowly on paraffin. Gordon (8) stated that many of the mycobacteria isolated from infected animal tissue utilized paraffin, and in other ways appear to be identical with soil strains. However, the possibility that these organisms were merely soil contaminants cannot be overlooked (9).

The effect of long-chain, aliphatic fatty acids on the growth and metabolism of *Mycobacteria* has been the subject of various investigations (10, 11, 12). Dubos (13), and Dubos and Davis (14), have recently studied the effect of oleic acid on the growth of these organisms in some detail. Since some investigators have claimed that fatty acids are intermediate or end-products of hydrocarbon oxidation by bacteria, it was thought of interest to determine the effect of long-chain hydrocarbons on the oxidative metabolism and growth of representative strains of pathogenic Mycobacteria.

EXPERIMENTAL

Materials and Methods

Four strains of pathogenic *Mycobacteria* were used in this investigation, the H37Rv (virulent) and H37Ra (avirulent), obtained from Mr. W. Steenken, Trudeau Laboratories, Saranac, N. Y.; the Sigerson (avian) obtained from Dr. W. Feldman, Mayo Clinic, and a BCG strain.

*Present address: Department of Bacteriology, Iowa State College, Ames, Iowa.

For oxidative metabolic studies, the organisms were grown in Tween 80—bovine serum fraction V (albumin) medium (15) distributed in 100 ml. amounts in 500 ml. cotton-plugged Erlenmeyer flasks. Each flask was inoculated with 4 ml. of a 7–10 day old culture of the appropriate organism and incubated at 37.5°C. for 7–10 days. At the end of this period, 80–90 ml. of supernatant fluid were withdrawn from each flask by a pipette. The remaining fluid, with the organisms, was transferred to 250 ml. centrifuge bottles and centrifuged at 2000 r.p.m. for 20 min. The supernatant fluid was then withdrawn by pipette and the bacterial deposit was resuspended in 30 ml. of $M/15$ phosphate buffer, pH 6.8 or 7.4, and recentrifuged. This washing procedure was performed twice. The organisms were then kept in the ice chest at 5°C. until needed. The H37Ra and Sigerson strains could be harvested and kept at ice chest temperature for 5–7 days without deterioration, the H37Rv and BCG strains slowly lost ability to oxidize various substrates when left for periods longer than 72 hr.

Immediately prior to use in oxidative studies, the organisms were resuspended in such amounts of $M/15$ phosphate buffer, pH 6.8 or 7.4, that each ml. of suspension contained 13–17 mg. of bacterial cells on a dry weight basis. Dry weights were determined in the following manner:

Forty ml. of a 7–10 day culture of organisms were transferred to a 100 ml. centrifuge tube and centrifuged at 4500 r.p.m. for 1.5 hr. The supernatant fluid was then withdrawn by pipette and the organisms, resuspended in 5 ml. of $M/15$ phosphate buffer, pH 7.4, containing 0.1% Tween 80, were transferred quantitatively by means of capillary pipette, to tared Wassermann tubes. The organisms were again centrifuged as above, resuspended and washed twice with $M/15$ phosphate buffer, pH 7.4, and finally with 7 ml. of 95% ethyl alcohol. After pipetting off the alcohol, the organisms in the Wassermann tubes were placed in a vacuum oven at 65°C. for 24 hr., and the weight of the organisms calculated by difference. At the same time, the original culture was serially diluted and the optical density of each dilution determined in the Coleman Junior Clinical Spectrophotometer. A straight line relationship was obtained when optical density of the diluted culture was plotted against dry weight of organisms. After this calibration, optical densities were determined on each culture, and dry weights calculated from the densities.

This procedure was performed with each of the 4 cultures used. The curve for the Sigerson strain was virtually superimposable upon the BCG curve. It was found that the blank used in the Coleman reading could be either culture filtrate, fresh culture medium, or phosphate buffer, with no significant difference in result at a wavelength of 650 mμ.

Oxygen uptake was measured in the Warburg apparatus, under air, at 37.5°C. in the usual manner. There was added to each Warburg vessel, 1.0 ml. of bacterial suspension 0.05 ml. of hydrocarbon and 0.95 ml. of $M/15$ phosphate buffer, pH 6.8 or 7.4. *n*-Octadecane, which is solid at room temperature, but liquid at 37.5°C., was added to flasks in the melted state. The sidearms contained 0.5 ml. of buffer and the insert 0.2 ml. of 10% KOH, with folded analytical filter paper. Control vessels containing no hydrocarbon received an additional 0.05 ml. of buffer. CO_2 determinations were performed according to the three flask method described by Umbreit *et al.* (16).

The vessels were allowed to equilibrate for 15 min. with the manometers open to air. The shaking speed was 120–130 excursions/min. and the amplitude was 3 cm. Slower

shaking and smaller amplitudes decreased the O_2 uptake slightly. Higher shaking speeds did not alter the results. In general, reactions were allowed to proceed for 5 hr.

All the chemically defined hydrocarbons were purchased from the Connecticut Hard Rubber Company, New Haven, and were guaranteed to be at least 95 mole-% pure.

RESULTS

Effect of Saturated and Unsaturated Hydrocarbons on O_2 Consumption

It was noted that, with all hydrocarbons of chain length greater than 8 carbons, the organisms migrated almost completely to the Buffer-hydrocarbon interface, leaving only a slightly turbid aqueous layer. In the case of the octenes and octane, the organisms clumped into a large, pea-sized, yellowish floating mass of rubbery consistency. Hydrocarbons containing less than 8 carbons did not exhibit this effect. The migration of acid-fast bacilli to the hydrophobic layer has been studied in detail by Reed and Rice (17), among others.

In general, the Sigerson (avian) strain showed considerably greater increases in O_2 uptake under the influence of various hydrocarbons than did the other 3 strains studied. However, the 14, 16, and 18 carbon saturated and 1-unsaturated hydrocarbons greatly increased the O_2 consumption of all 4 strains of organism. Figs. 1–4, which are representative experiments, show the extent of this effect.

With *n*-decane and *n*-dodecane, the BCG, Sigerson and H37Ra strains showed definite, increased O_2 uptake, though somewhat less than with the higher hydrocarbons. On the other hand, the H37RV strain, respiring in the presence of the 10 and 12 carbon saturated compounds, showed slight or no increase in O_2 consumption.

In the presence of 1-dodecene, H37Ra respired as well as in the presence of the higher saturated and unsaturated homologs, while the avian strain showed a somewhat smaller increase in O_2 consumption. Both the BCG and H37Rv strains were inhibited by 1-decene and 1-dodecene. The degree of stimulation of the H37Ra and Sigerson strains by 1-decene was considerably less marked than with the higher homologs.

n-Octane caused either slight inhibition or slight stimulation of the respiration of all 4 strains, whereas 1-octene and 2-octene and 1-heptene markedly inhibited the respiration of all organisms. It is possible that the effect of the 8 carbon compounds may be due to the clumping of the organisms in the experimental flasks.

When respiring in the presence of *n*-hexane, the BCG strain exhibited marked stimulation, whereas the other 3 strains were, for the most part, unaffected. 1-Hexene had no effect on the respiration of any of the strains.

The above results are summarized in Table I. It should be mentioned that

the hydrocarbons alone, or hydrocarbon plus heat-killed tubercle bacilli showed no O_2 uptake.

Effects of Miscellaneous Hydrocarbons on Oxidative Metabolism

Paraffin wax slightly increased the respiration of all 4 strains, the BCG strain being least affected. Ligroin (B.P. 60°–90°), *n*-heptane (B.P. 90°–100°), petroleum ether (B.P. 40°–50°), and Squibb Heavy Mineral Oil, either had no effect or decreased the O_2 uptake. Xylene and toluene completely inhibited the O_2 uptake of all strains.

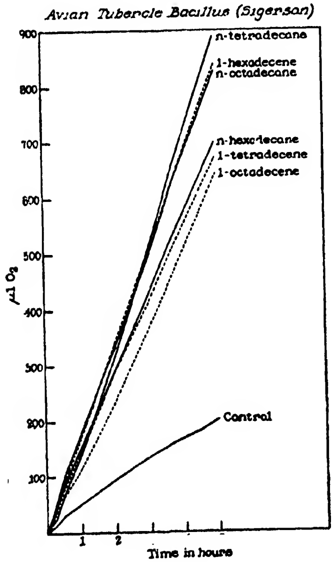


FIG. 1

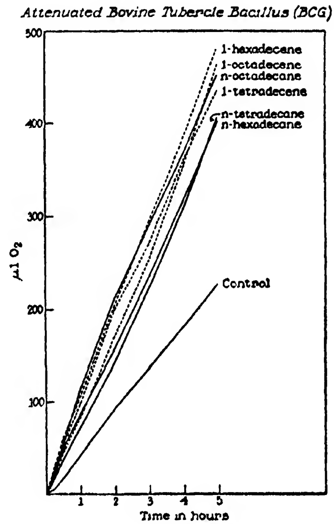


FIG. 2

FIG. 1. Effect of 40 mg. of various hydrocarbons on O_2 uptake of avian tubercle bacillus.

FIG. 2. Effect of 40 mg. of various hydrocarbons on O_2 uptake of BCG strain of tubercle bacillus.

Determination of RQ

n-Tetradecane was the substrate used for determination of the RQ. The theoretical RQ for a hydrocarbon completely oxidized to CO_2 and H_2O is 0.65. RQ values of 0.83 and 0.72 were noted for the BCG and H37Ra strains, respectively. If the hydrocarbon is being oxidized, this would suggest that intracellular decarboxylation mechanisms are being stimulated as well. The H37Rv strain gives an RQ of 0.67, a value indicating complete oxidation of the substrate, while the Sigerson strain gives an RQ value of 0.48, indicating approximately 75% oxidation of the substrate. Endogenous RQ measurements varied from 0.92 to 1.0 for all strains. It is realized that no definitive information as to the course of metabolism can be secured from RQ values alone.

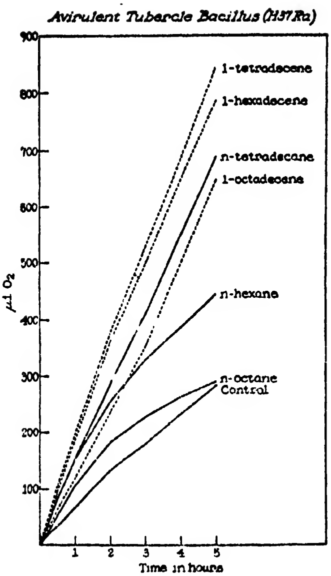


FIG. 3

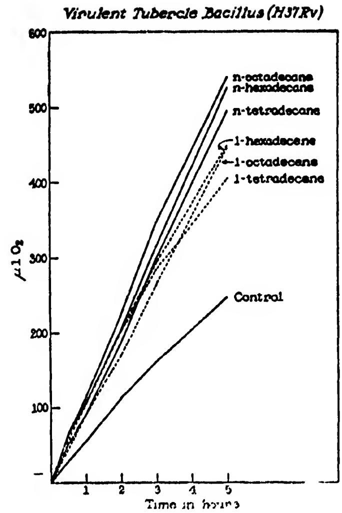


FIG. 4

FIG. 3. Effect of 40 mg. of various hydrocarbons on O_2 uptake of H37Ra strain of tubercle bacillus.

FIG. 4. Effect of 40 mg. of various hydrocarbons on O_2 uptake of H37RV strain of tubercle bacillus.

TABLE I

Effect of Various Hydrocarbons on the Respiration of Washed Mycobacteria

	Hydrocarbons																
	Control, no hydrocarbon	*n*-Hexane	*n*-Octane	*n*-Decane	*n*-Dodecane	*n*-Tetradecane	*n*-Hexadecane	*n*-Octadecane	1-Hexene	1-Heptene	1-Octene	2-Octene	1-Decene	1-Dodecene	1-Tetradecene	1-Hexadecene	1-Octadecene
Sigerson	−200	−231	−243	−616	−650	−720	−700	−706	−238	−100	−143	−29	−300	−365	−571	−600	−580
H37Ra	−280	−380	−257	−373	−512	−597	−562	−595	−388	−180	−215	−160	−398	−550	−659	−722	−561
BCG	−264	−374	−230	−317	−366	−440	−487	−485	−304	−126	−120	−78	−249	−184	−371	−440	−440
H37Rv	−266	−301	−226	−329	−322	−540	−545	−565	−274	−241	−151	−45	−239	−189	−377	−435	−499

The figures given are the average of O_2 uptake in μl. of numerous experiments. The duration of the experiments was 5 hr. Each cup contained 1 ml. bacterial suspension. 0.95 ml. $M/15$ PO_4 buffer, pH 6.8 or 7.4, and 0.2 ml. 10% KOH in well.

Effect of Two Long-Chain Alcohols on the Oxidative Metabolism

Oxygen uptake of the 4 strains was markedly stimulated by both cetyl and stearyl alcohols when flakes of these compounds were added to Warburg vessels. The O_2

uptake was considerably greater with cetyl alcohol than with stearyl alcohol, the Q_{O_2} being tripled in many cases.

Attempts to Isolate Acids as End Products of Hydrocarbon Metabolism

After the bacteria had acted upon *n*-tetradecane for 5 hr., the suspension of organisms was centrifuged for 1 hr. at 4500 r.p.m. The supernatant was removed and made acid (pH 3) with 10 *N* H_2SO_4. An aliquot portion was placed in the Kirk all-glass micro steam distillation apparatus. Five 10 ml. portions of distillate were collected. Titration with 0.01 *N* NaOH showed no acid present in the distillate.

Acidification of the reaction supernatant with 10 *N* H_2SO_4 and extraction with three 3 ml. portions of ether yielded no ether-soluble acids.

Attempts to Grow Tubercle Bacilli in Media Containing Hydrocarbon as Energy Source

A basal medium containing the salt composition suggested by Dubos and Middlebrook (15) was used. This was supplemented by 0.1% NH_4NO_3 or $(NH_4)_2SO_4$. The medium was dispensed in 5 ml. amounts into metal-capped potato tubes and autoclaved at 15 lb. pressure for 15 min. The hydrocarbons used, 14–18 C saturated and unsaturated, were autoclaved separately at 10/10 and added aseptically in 0.3 ml. amounts to the tubes. Tubes were inoculated with a 2×10^{-4} dilution of a 7 day old Tween 80—albumin culture and incubated at 37.5°C.

No growth was obtained in the basal medium + hydrocarbon, even after 4 weeks incubation. Two-tenths per cent asparagin was then added to the medium in place of the ammonium salts. This basal-asparagin medium supported satisfactory minimal growth.

With the Sigerson strain of tubercle bacilli, coarse, granular bottom growth appeared in 2 days and increased in amount at least up to 6 weeks in tubes containing *n*-tetra- and hexadecane and 1-octadecene. On the other hand, growth in the asparagin-basal medium without hydrocarbon appeared only after 6 days and remained minimal throughout the 6 weeks of the experiment.

No growth was obtained using *n*-octadecane and 1-tetra- and hexadecene.

Similar experiments with the H37Ra strain failed. No growth was obtained in any of the tubes containing hydrocarbons, though there was minimal growth in the control tubes.

Since the possibility existed that the hydrocarbons were dissimilated to long-chain fatty acids, and since serum albumin is known to neutralize the toxicity of higher fatty acids for *Mycobacteria*, bovine albumin (serum fraction V) was added aseptically in a final concentration of 0.5% to the asparagin-containing control medium. When hydrocarbons were added to this medium, good growth occurred with *n*-tetra-, hexa- and octadecane, but growth was no better than in the basal-asparagin-albumin medium. No growth was obtained in media containing the unsaturated 14–18 carbon hydrocarbons.

It would thus appear that, with the system used, the hydrocarbons tested

do not support the growth of the H37Ra strain of tubercle bacillus. Similar results were obtained with the H37RV strain.

DISCUSSION

It is obvious from the foregoing that certain hydrocarbons exert a profound effect upon the oxidative metabolism of tubercle bacilli. In many cases, apparently the respiration of the *Mycobacteria* studied is more stimulated by hydrocarbons than by any of the more usual carbon sources. The increase in O_2 consumption due to the 14, 16, and 18 carbon hydrocarbons is much larger than the increase seen, for example, with glucose or glycerine.

There are several possible explanations for the stimulation observed. Most obvious is the possibility that the hydrocarbons are utilized as energy sources by the organisms. R. Q. measurements and the fact that no end-products (*e.g.*, aldehydes, acids) have been found in the reaction mixture after 5 hr. of incubation would tend to indicate that the compounds are almost completely oxidized. However, only in the case of the Sigerson (avian strain) has stimulation of growth been noted in the presence of hydrocarbons.

A clear-cut answer to the question of actual utilization could presumably be supplied by the use of hydrocarbons containing isotopic carbon and measurement of the liberated CO_2 for isotope content. Work along these lines is being done.

It is quite possible that the results observed are caused by the dispersing or congregating effects of the hydrocarbons on the bacteria, thus increasing or decreasing the respiration by changing the total free surface of the cells. Lending credence to this hypothesis is the fact that, in the case of the 8-carbon compounds, which inhibit respiration, a marked congregating action of the hydrocarbons is observed. On the other hand, those hydrocarbons which stimulate the respiration quite obviously wet the surface of the bacteria, and presumably allow for dispersion.

These possibilities have been presented as being most likely to account for the mode of action of the hydrocarbons. Undoubtedly there are others, and further work is being done to elucidate the mechanism of the effect.

SUMMARY

1. Higher hydrocarbons and alcohols have been tested for their effect on the oxygen uptake of tubercle bacilli. The 14, 16, and 18 carbon saturated and unsaturated hydrocarbons, in particular, greatly increase the O_2 uptake of 4 strains of pathogenic mycobacteria studied. Cetyl and stearyl alcohols have an anlogous effect. Fatty acids are apparently not end-products of the reaction.

2. *n*-Tetradecane, *n*-hexadecane and 1-octadecene stimulate the growth of the avian strain. The other strains tested are apparently unable to utilize the hydrocarbons as energy source for growth.

3. RQ measurements are compatible with the hypothesis that hydrocarbons

are almost completely oxidized to CO_2 and H_2O. Other possible modes of action of the hydrocarbons are discussed.

Acknowledgment

The author wishes to express his gratitude to Dr. René J. Dubos, in whose laboratory this work was performed, for his many valued suggestions and criticisms.

REFERENCES

1. ZoBell, C. E., *Bact. Revs.* **10,** 1 (1946).
2. Söhngen, N. L., *Zentr. Bakt. Parasitenk. Abt. II,* **37,** 595 (1913).
3. Büttner, H., *Arch. Hyg.* **97,** 12 (1926).
4. Haag, F. E., *Zent. Bact. Parasitenk. Abt. II,* **71,** 1 (1927).
5. Jensen, H. L., *Proc. Linnean Soc. N. S. Wales* **59,** 19 (1934).
6. Bushnell, L. D., and Haas, H. F., *J. Bact.* **41,** 653 (1941).
7. Haas, H. F., Thesis, Kansas State College, Manhattan, Kansas, 1942.
8. Gordon, R. E., *J. Bact.* **34,** 617 (1937).
9. Gordon, R. E., personal communication.
10. Cutinelli, C., *Chem. Abstracts* **34,** 5110 (1940).
11. Franke, W., and Schillinger, A., *Biochem. Z.* **316,** 313 (1944).
12. Bergström, S., Theorell, H., and Davide, H., *Nature* **157,** 306 (1947).
13. Dubos, R. J., *Proc. Soc. Exptl. Biol. Med.* **63,** 56 (1946).
14. Dubos, R. J., and Davis, B. D., *J. Exptl. Med.* **83,** 409 (1946).
15. Dubos, R. J., and Middlebrook, G., *Am. Rev. Tuberc.* **56,** 334 (1947).
16. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric Techniques and Related Methods for the Study of Tissue Metabolism. Burgess Publishing Co., Minneapolis, 1945.
17. Reed, G. B., and Rice, C. E., *J. Bact.* **22,** 239 (1931).

[Reprinted from THE JOURNAL OF LABORATORY AND CLINICAL MEDICINE, July, 1949]
Vol. 34, No. 7, pp. 1023-1026]

PRESERVATION OF VIRUSES IN A MECHANICAL REFRIGERATOR AT −25° C.

BY PETER K. OLITSKY, M.D., JORDI CASALS, M.D., DUARD L. WALKER, M.D.,* HAROLD S. GINSBERG, M.D., AND FRANK L. HORSFALL, JR., M.D.

(*From the Laboratories and the Hospital of The Rockefeller Institute for Medical Research*)

(Received for publication, May 12, 1949)

As a routine, many laboratory workers who employ viruses in investigations or in diagnostic procedures store infectious material in the frozen state at about −70° C. in cabinets[1] that are refrigerated with solid carbon dioxide. Although this way of preservation is useful and effective, it has some disadvantages. Of these, the most important are: (1) the necessity for a continuous supply of solid CO_2; (2) the high annual cost of solid CO_2; and (3) the acidity caused by the diffusion of CO_2 into specimens. In many geographical areas a regular supply of solid CO_2 cannot be obtained. In areas where the refrigerant is readily available, the cost of constantly maintaining low temperatures with solid CO_2 is considerable, even when efficient cabinets are employed (the outlay may run to $100 to $200 per cubic foot of storage space per year). Although diffusion of CO_2 into specimens can be prevented by the use of sealed hard-glass containers, this procedure is cumbersome and inconvenient.

Subfreezing temperatures now can be maintained with a number of commercially available mechanical refrigerators. Certain electrically operated ones maintain temperatures in the range attained with cabinets cooled with solid CO_2, but their initial cost is high and their storage capacity not large. Other less costly refrigerators can maintain temperatures in the range of −20 to −30° C. and of these many provide large storage space.

The preservation of viruses for prolonged periods at −70° C. has been studied in a number of instances[1-3] and it is now well known that most viruses retain infectivity for long intervals when properly stored at this temperature. However, relatively little is known as to the stability of viruses at −20 to −30° C. Melnick[4] found that Y-SK and Ph poliomyelitis viruses as well as the TO Theiler virus retained their original titers when stored for twelve months at −20° C. Under the same conditions the titer of Japanese B virus decreased by one thousand fold or more.

In the present investigation, infectivity titrations were carried out with a number of different viruses which were stored in an electrically operated mechanical refrigerator for periods up to twelve months at temperatures ranging from −20 to −30° C. The results indicate that all of the viruses studied can be

* Fellow in the Field of Virus Diseases of the National Research Council.

stored under these conditions for at least three months without causing any striking reduction in infectivity. With most of the neurotropic viruses, full infectivity was maintained for nine months. With the influenza-mumps group of viruses, infectivity was much diminished although still present after twelve months. It should be mentioned that during the study one refrigerator was once accidentally disconnected, and the other twice failed to operate properly. During these periods the temperature increased sufficiently so that all specimens were thawed for an interval of less than twenty-four hours.

EXPERIMENTAL

The following viruses were employed: Eastern equine encephalitis (Rockefeller Institute strain), Western equine encephalitis (Rockefeller Institute strain), Russian Far East encephalitis (No. 1 strain), Japanese B encephalitis (Nakayama strain), poliomyelitis (Lansing strain), poliomyelitis (MEF1 strain), influenza A virus (PR8 strain), influenza B virus (Lee strain), and mumps virus (Habel strain).

The neurotropic viruses were contained in suspensions of fresh, infected mouse brains or in frozen suspensions previously stored in a CO_2 cabinet. The suspensions stored were 10^{-1} dilutions of brain in either 10 or 50 per cent normal rabbit serum which were placed either in screw-capped nitrocellulose tubes or in sealed Pyrex ampules. Influenza and mumps viruses were contained in allantoic fluid obtained from infected chick embryos. One portion of each allantoic fluid was stored in the undiluted state, the other after dilution to 10^{-1} with normal horse serum. The allantoic fluids were all kept in screw-capped nitrocellulose tubes.

In every instance a number of specimens of each virus preparation were quickly frozen by partial submersion in a mixture of ethyl alcohol and solid CO_2 before they were placed in the refrigerator at -20 to $-30°$ C. At appropriate intervals one specimen of each frozen suspension was thawed rapidly in running tap water and an infectivity titration was performed. Titrations were carried out with serial decimal dilutions. With the neurotropic viruses the usual intracerebral technique in white mice was employed, and the LD_{50} was determined. In the case of influenza and mumps viruses the intra-allantoic technique in chick embryos was used, and the EID_{50} was determined by hemagglutination tests with allantoic fluids removed after an appropriate interval.

The refrigerators employed were commercially available models which had single stage compressor units and were capable of maintaining temperatures of $-30°$ C. on intermittent operation. One refrigerator had a storage capacity of 20 cubic feet, the other, 16.

The results of the titrations are presented in Table I. It will be noted that all of the viruses which were suspended in 50 or more per cent normal serum retained infectivity throughout the entire period of observation. It will also

be seen that the Russian Far East encephalitis virus and the MEF1 and the Lansing strains of poliomyelitis virus retained almost their original LD_{50} titers for a period of nine months. Melnick[4] also showed a similar, relative stability for other Lansing-type viruses. The Western equine encephalitis virus showed only a slight reduction in titer after nine months of storage and the titer of the Eastern equine encephalitis virus decreased only about 1.5 log units over

TABLE I

Effect of Storage at −20 to −30° C. on Infectivity of Viruses

Virus	Material Used	Suspended in	Original Titer LD_{50}	Titer After Storage: 3 Mo. (LD_{50})	6 Mo. (LD_{50})	9 Mo. (LD_{50})
Eastern equine.....	10^{-1} m.b.	50% n.r.s.	$10^{-9.0}$	$10^{-7.4}$	$10^{-7.2}$	$10^{-7.5}$
Western equine....	10^{-1} m.b.	50% n.r.s.	$10^{-8.3}$	$10^{-8.0}$	$10^{-7.8}$	$10^{-7.8}$
Russian Far East...	10^{-1} m.b. (fresh)	10% n.r.s.	$10^{-8.8}$	$10^{-5.5}$	$10^{-3.0}$	—
Russian Far East...	10^{-1} m.b. (fresh)	50% n.r.s.	$10^{-9.4}$	$10^{-9.4}$	$10^{-8.8}$	$10^{-8.6}$
Japanese B	10^{-1} m.b. (fresh)	50% n.r.s.	$10^{-8.5}$	$10^{-7.5}$	$10^{-4.2}$	$10^{-2.6}$
Poliomyelitis, Lansing	10^{-1} m.b.	50% n.r.s.	$10^{-2.5}$	$10^{-2.1}$	$10^{-2.2}$	$10^{-2.2}$
Poliomyelitis, MEF1	10^{-1} m.b. (fresh)	50% n.r.s.	$10^{-3.2}$	$10^{-2.9}$	$10^{-2.3}$	$10^{-3.3}$
			EID_{50}	3 Mo. (EID_{50})	6 Mo. (EID_{50})	1 Yr. (EID_{50})
Influenza A, PR8	10^{-1} all. fl.	90% n.h.s.	$10^{-8.8}$	$10^{-8.4}$	$10^{-5.0}$	$10^{-5.0}$
Influenza A, PR8	Undil. all. fl.	—	$10^{-8.3}$	$10^{-7.5}$	$10^{-4.7}$	$<10^{-3.0}$
Influenza B, Lee	10^{-1} all. fl.	90% n.h.s.	$10^{-7.8}$	$10^{-7.0}$	$10^{-4.5}$	$10^{-3.3}$
Influenza B, Lee	Undil. all. fl.	—	$10^{-7.8}$	$10^{-7.0}$	$10^{-3.5}$	$<10^{-2.0}$
Mumps	10^{-1} all. fl.	90% n.h.s.	$10^{-7.5}$	$10^{-7.0}$	—	$10^{-4.5}$
Mumps	Undil. all. fl.	—	$10^{-6.6}$	$10^{-5.7}$	—	$<10^{-1.0}$

m.b., Mouse brain suspension in normal rabbit serum.
n.r.s., Normal rabbit serum in saline solution.
all. fl., Allantoic fluid.
n.h.s., Normal horse serum.
EID_{50}, 50 per cent end point of virus titration in chick embryos.

the same period. However, the Japanese B encephalitis virus, although still infective (i.e., LD_{50} $10^{-2.6}$) after nine months, had decreased markedly in titer. This latter finding is in good agreement with the results reported by Melnick.[4]

It will be observed that influenza A, influenza B, and mumps viruses showed only slightly reduced EID_{50} titers after storage for three months. However, after six months of storage the titers of the influenza viruses had decreased by

more than 3 log units. This was true even in the case of the suspensions which had been prepared in 90 per cent normal horse serum. After storage for one year, none of the three viruses kept in undiluted allantoic fluid showed any evidence of infectivity, and all suspended in horse serum gave titers 3 to 4 log units lower than the original titers of the suspensions.

DISCUSSION

It appears evident that a number of neurotropic viruses can be preserved by storage at -20 to $-30°$ C. for periods of at least nine months without much loss of infectivity. Japanese B encephalitis virus seems to be the least stable of the neurotropic viruses tested; it showed a marked reduction in titer after storage for six months at this temperature. Influenza and mumps viruses exhibited a stability, under the conditions employed, which was approximately of the same order as that of Japanese B virus. Their infectivity titers also were considerably reduced following storage for six months at $-25°$ C.

It should be emphasized that of the viruses employed, those suspended in 50 to 90 per cent normal animal serum retained infectivity sufficient to permit recovery easily after storage for at least nine months. Moreover, when such concentrations of normal serum were used none of the virus suspensions showed any striking reduction in titer after three months of storage; only those of Eastern equine and Japanese B encephalitis exhibited a decrease of 1 or more log units. It also should be stressed that the results were obtained despite the fact that the viruses had been accidentally thawed once or twice for an interval of some hours.

For many virus studies a period of storage of three months is satisfactory. In laboratories in which a CO_2 refrigerated cabinet is not available, and in areas in which a supply of solid CO_2 cannot be obtained, electrically operated, mechanical refrigerators which maintain a temperature of -20 to $-30°$ C. may prove useful.

SUMMARY

Numerous viruses can be preserved by storage in an electrically operated mechanical refrigerator at -20 to $-30°$ C. The infectivity titer of a number of viruses did not diminish significantly after storage for three months. In spite of the fact that some showed a marked reduction in titer after nine months, some became inactivated if stored in the presence of an adequate amount of normal serum.

REFERENCES

1. Horsfall, F. L., Jr.: A Low Temperature Storage Cabinet for the Preservation of Viruses, J. Bact. **40:** 559, 1940.

2. Horsfall, F. L., Jr.: Neutralization of Epidemic Influenza Virus. The Linear Relationship Between the Quantity of Serum and the Quantity of Virus Neutralized, J. Exper. Med. **70:** 209, 1939.
3. Horsfall, F. L., Jr., and Curnen, E. C.: Studies on Pneumonia Virus of Mice. II. The Precision of Measurements in Vivo of the Virus and Antibodies Against It, J. Exper. Med. **83:** 25, 1946.
4. Melnick, J. L.: Storage of Mouse-Adapted Strains of Poliomyelitis Virus and of Japanese B. Encephalitis Virus at Subfreezing Temperatures, J. Infect. Dis. **79:** 27, 1946.

[Reprinted from THE JOURNAL OF BACTERIOLOGY, July, 1949, Vol. 58, No. 1, pp. 112–113]

ADDITIONAL PROPERTIES OF THE MEF1 STRAIN OF POLIOMYELITIS VIRUS, ESPECIALLY WITH REFERENCE TO ATTEMPTS AT CULTIVATION IN THE CHICK EMBRYO

BY ROBERT H. YAGER,* PETER K. OLITSKY, AND O. LAHELLE‡

(*From the Laboratories of the Rockefeller Institute for Medical Research*)

(Received for publication, April 5, 1949)

Successful cultivation in fertile hens' eggs has been reported of Theiler's virus, TO, FA, GDVII strains, but not of poliomyelitis virus, Lansing, Y-SK, Ph, and several other human strains (Riordan and Sa-Fleitas: J. Immunol., **56**, 263, 1947; Dunham and Parker: J. Bact., **45**, 80, 1943; and others). These findings on TO, FA, and GDVII and on Lansing viruses have been confirmed in this laboratory.

In view of the fact that multiplication in chick embryos is a distinct feature separating so-called murine from poliomyelitis viruses and since the MEF1 strain (Schlesinger, Morgan, and Olitsky: Science, **98**, 452, 1943) has not as yet been studied in this regard, the present report on such studies including certain other properties is presented.

Chick embryos, 7 to 11 days old, were inoculated with a 20 per cent MEF1 virus as a mouse brain suspension, 0.03 ml intracerebrally, 0.25 ml into the yolk sac, or 0.1 ml on the chorioallantoic membrane (C.A.). The membranes were then incubated for 7 to 11 days, the yolk sacs for 11 days, and the intracerebral series, 7 days. The C.A. or embryo (or both) and brain in 10^{-1} dilution were subinoculated intracerebrally in mice. Blind passage from the inoculated embryos to 2 or more series of normal chick embryos was made along with subinoculation in mice. The result was that MEF1 virus was found to be incapable of multiplying in chick embryos even intracerebrally. It is of interest that in one instance in the C.A.-inoculated series the membrane in 10^{-1} dilution induced paralyses in the mice. Although the mouse brain virus was identified by positive neutralization with Lansing antiserum, it was proved by passage that the virus did not multiply in the membrane but that it only persisted in the inoculum.

In addition, the MEF1 after a large number of mouse passages exhibits a higher LD_{50} titer after intracerebral inoculation in the Rockefeller Institute strain of mice, viz., 3.0 to 4.2, than it does in the Lansing strain, which after many more mouse passages still shows the LD_{50} titer not to exceed 3 and at times less than 2. An added factor is the uniformity with which these animals respond to low dilutions of MEF1 virus as compared with the irregularity of reactors to those of the Lansing strain. This offers an advantage for certain experiments with rodent-adapted poliomyelitis viruses.

*Lt. Colonel, V.C., U. S. Army.

‡ Fellow, Scientific Research Fund, Oslo, Norway.

[Reprinted from The Journal of General Physiology, March 20, 1949,
Vol. 32, No. 4, pp. 409–443]

ON THE ORIGINS OF DORSAL ROOT POTENTIALS*

By DAVID P. C. LLOYD and A. K. McINTYRE

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, October 11, 1948)

Observations of potential changes occurring in dorsal roots during activity of the spinal cord date from the experiments of Gotch and Horsley, commenced in 1888 and fully described in their Croonian Lecture of 1891 (18). Recent attempts at analysis and interpretation of such potential changes may be said to begin with the work of Barron and Matthews (1), who described a simple fluctuation of long duration, provoked by afferent stimulation and having negative electrical sign at the more centrally located of two leads placed on the central segment of an isolated severed dorsal root: the so called dorsal root potential. So far as this single potential change is concerned the observations of Barron and Matthews are substantially correct. During the past decade essential experimental confirmation has come from the work of Bonnet and Bremer (2, 3), Bremer and Bonnet (5), Bremer, Bonnet, and Moldaver (6), Dun and Feng (11), Eccles and Malcolm (16), and during the course of the present investigation.

In contrast to the general agreement hitherto found at the level of observation, there has been widespread divergence of opinion concerning interpretation, due in part to the lack of an adequate theoretical background and in part to the lack of an adequate description of the sequence of potential changes that constitute the dorsal root potential. The present account of dorsal root potentials in the main is concerned with those parts of the sequence that have not received due attention; it adds little to what is already known about that part of the sequence represented by the negative deflection of Barron and Matthews.

Procedure

Bullfrog and cat preparations have been employed. In the former entire dorsal roots were isolated for stimulation and recording, but in the latter it was necessary to segregate individual rootlets to obtain well defined root-cord junctions. The roots or rootlets, severed distally, were raised into an insulating medium (paraffin) and fitted with electrodes as desired. Recording leads routinely were placed on dorsal roots one close to, but not touching, the spinal cord, the other at a distance toward the severed end. Throughout the present discussion these will be designated the proximal

* Presented at the Atlantic City meeting of the American Physiological Society (21).

and distal leads respectively, and the sign of a potential change, as is customary in work with dorsal root potentials, will be expressed in reference to the proximal lead. When stimulating electrodes were applied to roots from which recordings were to be made they were placed near the severed end distal to the distal recording lead. Any root that was stimulated to carry an afferent volley will be designated the active root, others will be designated neighboring roots. Direct coupled amplification has been standard practice.

The Problem

Fig. 1 illustrates potential changes in bullfrog dorsal roots evoked by single dorsal root volleys and recorded, in A, from the active root and, in B, from a

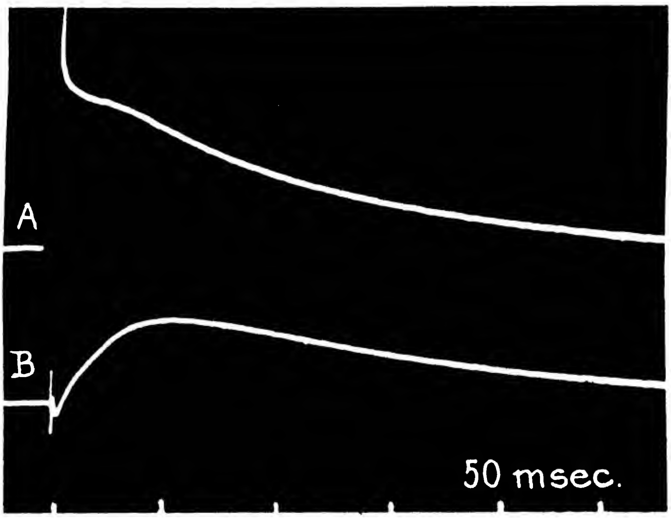


FIG. 1. Potential changes following single shock stimulation of a dorsal root and recorded by means of an electrode pair applied to the root stimulated (A) and to a neighboring root (B). Negativity at the root lead closer to the cord, in this and all other figures, is recorded upwards. Bullfrog preparation.

neighboring root of the same side. In record A, the tail of the large diphasic (positive-negative) spike potential of the traveling afferent volley may be seen, followed by a prolonged negative potential. In record B there is a sequence of potential changes culminating in a prolonged negative potential, that is the dorsal root potential of prior description. During the first 50 msec. following stimulation the relation between potential changes in the active and neighboring roots is not immediately apparent, but beyond that interval the changes in the two roots are comparable in size, duration, and electrical sign.

One must assume that the recording from an active root is of complex origin. Contributing to the over-all potential change will be: (*a*) potential gradients instituted in the dorsal root fibers by the stimulating current, a not insignifi-

cant factor considering the necessary proximity of electrodes placed four on a root, (*b*) after-potentials of the root fibers consequent upon conduction of a volley of impulses, a factor recognized by Woolsey and Larrabee (28), (*c*) gradients that might exist in the intramedullary segments of the stimulated fibers and which could propagate themselves back into the extramedullary segments, and (*d*) electrotonic potentials appearing in the root as the result of polarization of its intramedullary projection by the flow of current about active neurons.

Dorsal root potentials recorded in a neighboring root are less complex in origin than those of the active root for the direct consequences of the flow of stimulating current and of impulse conduction cannot be contributing fac-

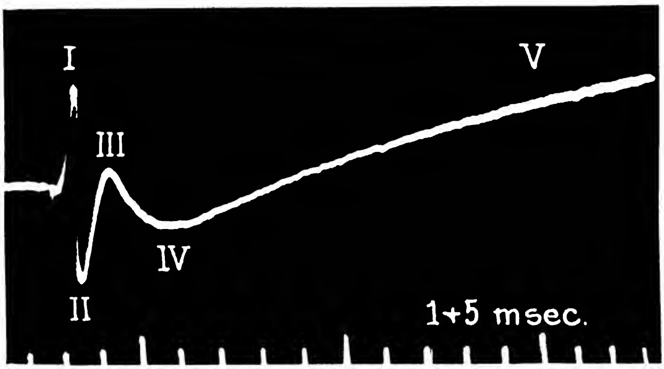


FIG. 2. Early course of dorsal root potential recorded, as in Fig. 1 B, from a root adjacent to that stimulated to show in greater detail the succession of deflections, D.R.I, II, III, IV and the beginning of D.R.V, which last is the dorsal root potential of prior description. Bullfrog preparation.

tors. A glance at record B of Fig. 1 reveals that there exist potential changes in a neighboring root additional to and anteceding the negative wave of the usual descriptions. Although these potential changes are to be found partially resolved in many of the previously published records of dorsal root potentials, they have been neglected except by Eccles and Malcolm (16) who regard them as artifacts introduced by contact between proximal recording lead and spinal cord. Such is not the case is the present recordings for which contact between lead and cord was scrupulously avoided. Furthermore the characteristic sequence is still recorded, but with the anticipated decrement, after the proximal lead is moved distally along the dorsal root.

Recorded by the use of higher oscillograph sweep speed, the early course of the dorsal root potential in a neighboring root may be seen in greater detail. Fig. 2, presenting such a recording, reveals that there are in all five deflections in the potential sequence. These deflections in order of temporal sequence are

to be designated D.R.I, D.R.II, D.R.III, D.R.IV, and D.R.V. Of these D.R.V is the negative wave of Barron and Matthews.

The dorsal root potential of a neighboring root is propagated electrotonically to the extramedullary segment of the contained primary afferent fibers from their intramedullary projections. The spinal cord constitutes a volume conductor within which the intramedullary projections of primary afferent fibers may be polarized by physical spread of current flow about active nervous elements. Such polarizations are the cause of electronic propagation into the extramedullary segment. Since the dorsal roots are raised into an insulating medium that extends to the cord junction, there is no significant *physical* spread of current from the cord into the extramedullary segment.

As a convenient simplification all the neurons within the cord are considered together as secondary neurons. This being done, one may state that polarization of the intramedullary segment must be due to either (*a*) the activity of primary afferent fibers or (*b*) secondary neurons. Now it is immediately obvious that D.R.I, II, and III together bear some relation to the triphasic intramedullary spike potential (17) of a primary afferent volley occupying the intramedullary segment of the stimulated dorsal root fibers. For this reason their origin may be ascribed with confidence to activity of primary afferent fibers. Secondary neurons clearly are responsible for D.R.V, as was first suggested by Bonnet and Bremer (2). Concerning the general properties of D.R.V the experimental description of Barron and Matthews is quite adequate. It remains then to clarify by experiment the origins and general properties of D.R.IV.

General Properties of D.R.IV

No *a priori* judgment may be made concerning the intramedullary activity responsible for the appearance, in a neighboring dorsal root, of the D.R.IV deflection. There exist three possible origins of that activity: (*a*) primary afferent fibers, (*b*) secondary neurons, and (*c*) both primary afferent fibers and secondary neurons. An advantageous means for study of D.R.IV is to compare its behavior in varied circumstances of stimulation, on the one hand with that of the D.R.I, II, III complex of primary afferent fiber origin, and on the other hand with that of D.R.V relating to secondary activity.

Antidromic Stimulation of Ventral Roots.—It is well known that a dorsal root potential of the type resembling D.R.V can be recorded, in the frog preparation, following antidromic stimulation of the motoneurons (1, 16). In Fig. 3 may be found records of dorsal root potentials evoked by stimulation with single shocks of an ipsilateral dorsal root (A and C), and of a ventral root (B and D), ipsilateral to, and of the same segment as, the dorsal root used for recording. Amplification was adjusted so that D.R.V and the antidromically evoked

potential that mimics it, would be of comparable recorded magnitude. At the left of Fig. 3 it can be seen that the two potential changes are reasonably comparable in general outline. In the early course of these potential changes, however, there are distinct differences seen to better advantage in C and D of Fig. 3, recorded with faster sweep speed. Present in record C, as the result of dorsal root stimulation is the characteristic sequence of deflections, D.R.I to V. By contrast the dorsal root potential consequent upon antidromic ventral root stimulation reveals between stimulus artifact and the onset of the prolonged negative deflection, some 12 msec. later, no change in the electrical state of the dorsal root. Thus the appearance in a dorsal root of a deflection re-

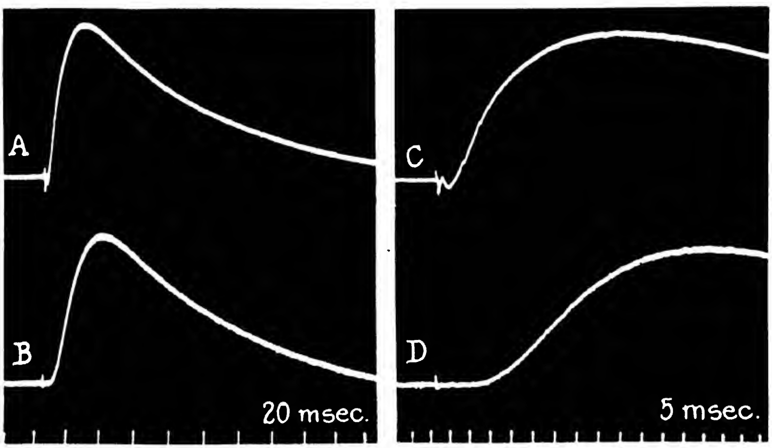


FIG. 3. Dorsal root potentials recorded, at two sweep speeds, following orthodromic stimulation of an adjacent dorsal root (A and C) and following antidromic stimulation of a ventral root (B and D). Bullfrog preparation.

sembling D.R.IV is not a necessary prelude to the appearance of a deflection resembling D.R.V. The observation demonstrates that activity in motoneuron somata, admittedly of a kind that results in a D.R.V-like wave, does not also polarize primary afferent fibers in the D.R.IV tempo, and suggests therefore that collaterals rather than somata may be responsible for D.R.IV.

Stimulation of Ipsilateral and Contralateral Dorsal Roots.—It is known from the observation of Barron and Matthews that the latency of D.R.V is longer when dorsal root potentials are recorded from a root contralateral to that stimulated than when recorded from an ipsilateral root. Fig. 4 illustrates a repetition of the observation to examine the antecedent potential deflections following ipsilateral (A) and contralateral (B) dorsal root stimulation. In each instance D.R.I, II, and III are present; so too is D.R.IV. As expected

the latency for D.R.V is widely different in the two recordings.[1] Despite this fact, virtually an identical temporal sequence exists between the D.R.I, II, III complex and D.R.IV, indicating the close association between these potential changes in circumstances that reveal a variable relation between D.R.IV and D.R.V.

Influence of Volley Size.—Fig. 5 presents a series of recorded dorsal root potentials arranged in order of increasing strength of stimulation. Record A of Fig. 5 was obtained by the use of a stimulus just over threshold strength; that

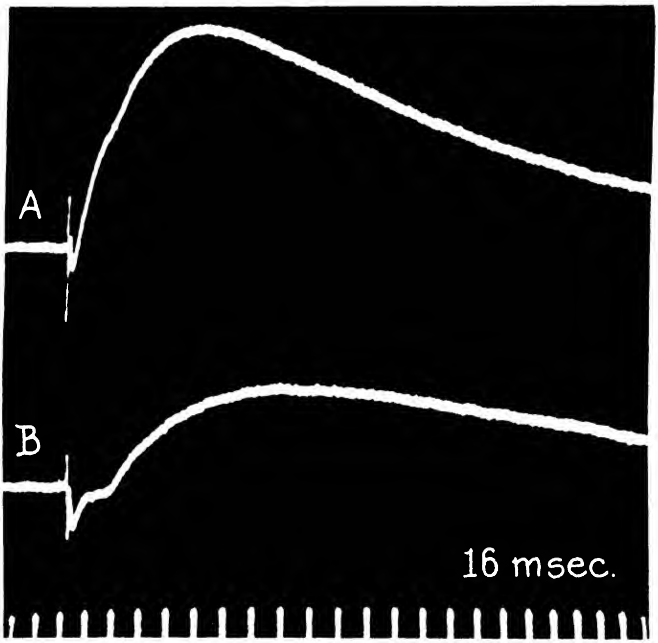


Fig. 4. Dorsal root potentials evoked by stimulation of an adjacent dorsal root (A) and of a contralateral dorsal root (B). Bullfrog preparation.

for record J was maximal. Dorsal root potentials evoked by contralateral dorsal root volleys were employed to avoid significant incursion of D.R.V upon D.R.IV with consequent distortion of the latter. Record A of Fig. 5 demonstrates that D.R.IV appears with D.R.I, II, III as threshold for dorsal root

[1] It is not possible to define with precision the latency of D.R.V. In ipsilateral recording, as may be seen in subsequent experiments, D.R.V would appear to begin as early as the peak of D.R.IV. Indeed, by interrupting the course of D.R.IV, D.R.V may determine in part the location of its peak. As an aside, recordings such as Fig. 4 A and Fig. 1 B, showing obvious discontinuities on the rising phase of D.R.V suggest that the potential change is not as simple as sometimes supposed (*cf.* also reference 11).

stimulation is exceeded, and records B to J show that the first four deflections of dorsal root potential increase in parallel with increase in volley size. In contrast to this parallel behavior D.R.V appears only after the antecedent deflections are well developed.

Experiment has shown that the clear-cut separation between the first four deflections of the dorsal root potential and the fifth that is obtained by varying the strength of contralateral dorsal root volleys is not so easily reproduced when ipsilateral dorsal root volleys are substituted for contralateral volleys. The fact is not surprising in view of the ever-present difficulties involved in segregat-

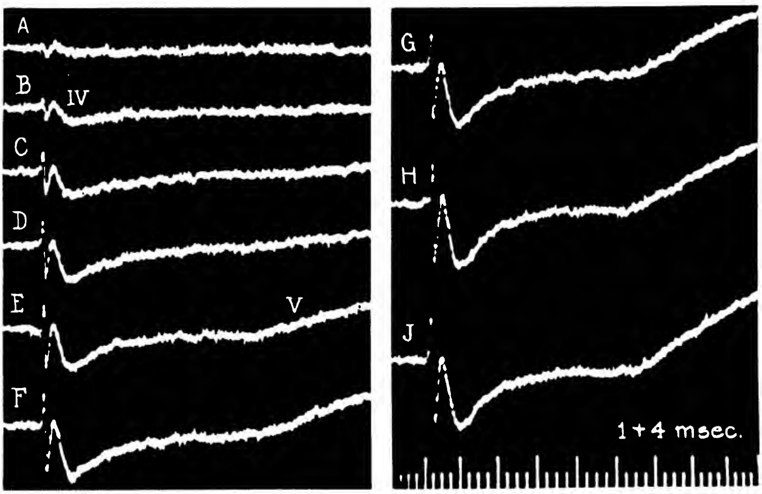


FIG. 5. Dorsal root potentials recorded, as in Fig. 4 B, from a root contralateral to that stimulated. Between each observation, A to J, stimulus strength was increased, that employed for A being just above dorsal root threshold, that for J being maximal. Sweep speed was such that only the onset of D.R.V appears in the recording. Bullfrog preparation.

ing one from another the various ipsilateral responses evoked by dorsal root stimulation (*cf.* reference 20, page 421).

When seen virtually free from interference, as in A, B, and C of Fig. 5, D.R.IV, in the bullfrog preparation, appears as a simple positive wave characterized by a rising phase of about 2 msec. duration and an approximately exponential decay to half-value in about 5 msec. It would seem a reasonable assumption that D.R.IV recorded from an ipsilateral root would exhibit a similar time course if, on recording, it were possible to divorce it from all interference from D.R.V. A number of experimental means have been tried, the most satisfactory being by the imposition of an asphyxial block. This is a simple procedure when studying the decapitate cat preparation, less so when employ-

ing the bullfrog preparation, due largely to the easy reversibility of the process in the former preparation.

Effect of Asphyxia on Dorsal Root Potentials.—In the experiment illustrated by Fig. 6, records of dorsal root potentials in a neighboring root were taken at regular intervals during a bout of asphyxia sufficiently prolonged to remove all trace of D.R.III, IV, and V. Representative records from the experiment have

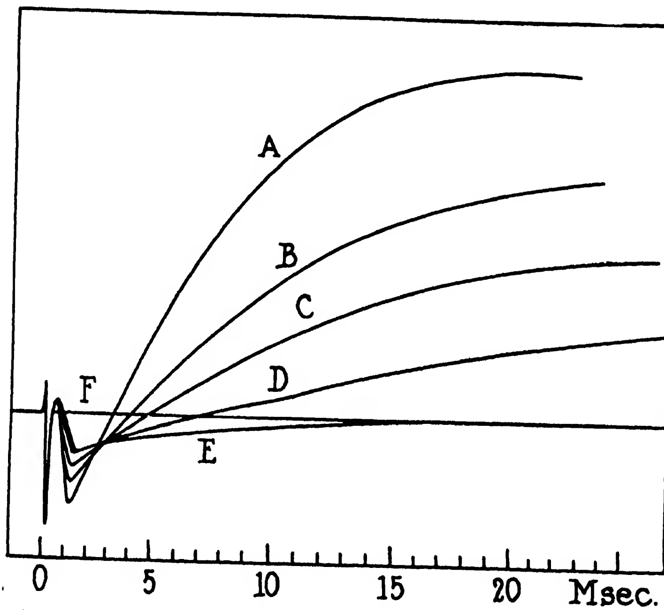


FIG. 6. Modification by asphyxia of dorsal root potentials recorded on a root adjacent to that stimulated. Cat preparation. A, normal dorsal root potential. E stage at which production of D.R.V is blocked (see also Fig. 7 B). F, stage at which only D.R.I and II appear. In the original records A to C, a small and progressively decreasing dorsal root reflex appeared; it has been omitted in reproduction. Details of the asphyxial block of dorsal root reflexes may be seen in Fig. 7.

been superimposed by tracing to illustrate successive stages of the asphyxial change, A being the normal dorsal root potential, E being the stage at which D.R. I to IV are still present but no trace of D.R.V remains, and F being the "final" stage in which only D.R.I and II are recorded. Thus of the five deflections D.R.I and II are the most resistant to asphyxia, D.R.III and IV are less so, and D.R.V is the most labile.

Fig. 7 contains actual records from another preparation of the normal dorsal root potential (A), and of the dorsal root potential at the stage of asphyxia in which D.R.V is removed. It is interesting in passing to note that the dorsal

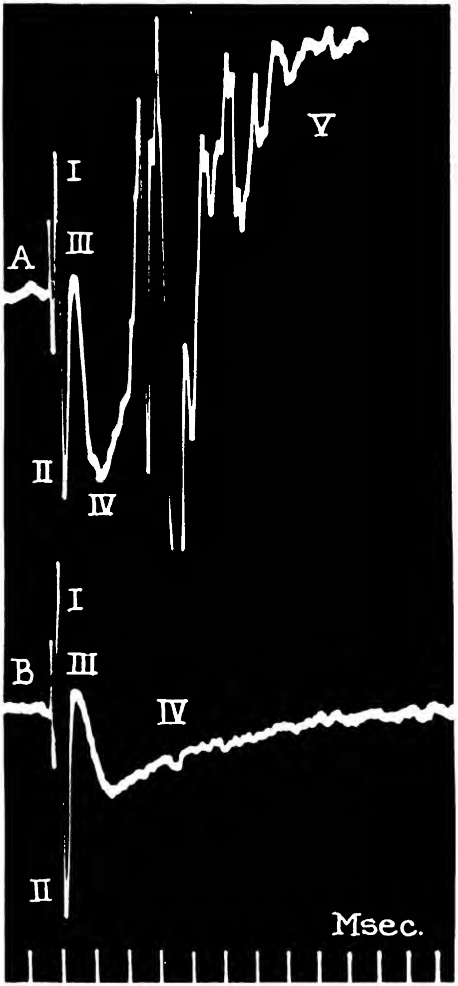


FIG. 7. Dorsal root potential recorded from a root adjacent to that stimulated. Cat preparation. A, normal, showing in this instance a large dorsal root reflex recorded in approximate diphasicity. The successive deflections of the dorsal root potential are identified by Roman numerals. B, to illustrate dorsal root potential as modified by asphyxia, the stage being comparable to that of Fig. 6 E. Deflections D.R.I, II, and III are not demonstrably altered from the normal. D.R.V and the dorsal root reflex have not been produced. D.R.IV is reduced and possibly slowed but on this latter point an unequivocal statement cannot be made. In this preparation, and in the stage of asphyxia represented in record B, it was seen that D.R.IV deflections evoked by simultaneous combination of two dorsal root volleys summed exactly. In accordance with the present analysis record B illustrates that part of the dorsal root potential referable to the activity of primary afferent fibers.

root reflex discharge, recorded diphasically on the rising phase of D.R.V in the normal condition, disappears along with D.R.V.

Seen cleared from D.R.V during the course of asphyxia, D.R.IV, in the cat, appears as a simple positive wave characterized by a rising phase of from 0.8 to 1.0 msec. in duration, and an approximately exponential decay to half-value in 3 msec., 2.6 to 3.5 being the observed variation in the series of experiments under consideration.

Summation and Occlusion of D.R.IV and D.R.V. -When two volleys of impulses enter the spinal cord through separate dorsal roots or rootlets, the dorsal root potentials being recorded in a third, and when the recorded dorsal root potentials evoked by the two volleys severally and together are compared, it is found that D.R.IV and D.R.V behave in a widely different manner. Results obtained from the bullfrog preparation and the cat preparation are qualitatively comparable.

If the two dorsal root volleys reach the spinal cord together over roots of opposite sides, the recorded D.R.IV deflection is equal, or very nearly equal, to the sum of the D.R.IV deflections resulting from the two volleys in isolation, whereas D.R.V suffers almost total occlusion. In Fig. 8 this finding is illustrated from an experiment with the bullfrog preparation. Represented by the broken lines (I) and (C) are the dorsal root potentials recorded, above with a fast sweep, below with a slow sweep of the oscillograph spot, following ipsilateral and contralateral dorsal root stimulation. The solid line (I + C) represents the dorsal root potential following combined stimulation.

If the two dorsal root volleys reach the cord both by roots ipsilateral to the root employed for recording, D.R.IV exhibits a degree of occlusion, greater in the cat preparation than in the bullfrog preparation, but at no time comparable to the subsequent occlusion of D.R.V. Fig. 9, constructed in the same fashion as Fig. 8, illustrates an experimental result in a bullfrog preparation. In the lower part of Fig. 9, it will be seen that the D.R.V deflection following simultaneous combination of two ipsilateral dorsal root volleys is little more than the greater of the two D.R.V deflections caused by the volleys in isolation, whereas there is only a slight failure of summation on the part of the D.R.IV deflections (upper part of Fig. 9).

In the normal cat preparation, when dorsal rootlets of the same side are stimulated in simultaneous combination and the dorsal root potential resulting is recorded from a third rootlet on the same side it is usual to find a degree of occlusion in D.R.IV potential. The deficit, as seen in the upper part of Fig. 10, is typically greater in the cat preparation than in the bullfrog preparation. If now the cat preparation be subjected to asphyxia until the stage is reached wherein D.R.V is abolished, but D.R.IV is still present, occlusion of D.R.IV disappears to be replaced by exact summation. This change in behavior is illustrated by Fig. 10, in which summation of D.R.IV in the normal state (above) and in the partially asphyxiated state (below) may be compared.

Conclusion as to the Origin of D.R.IV.—It seems quite clear that D.R.IV may have origin in more than one type of intramedullary activity. The fraction

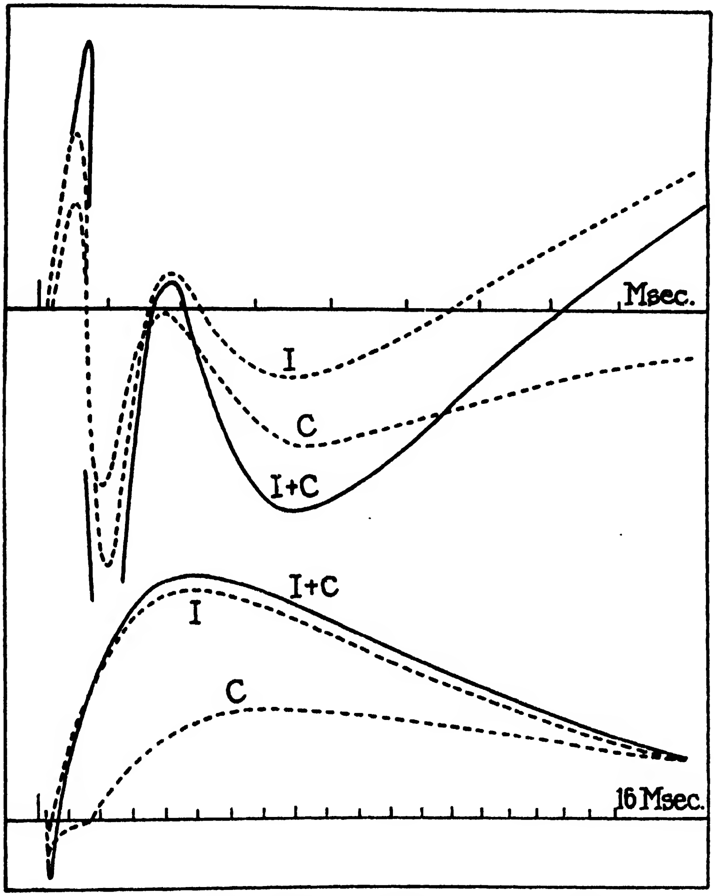


FIG. 8. Above, early course of dorsal root potentials elicited by stimulation of an ipsilateral adjacent dorsal root (I); by stimulation of a contralateral dorsal root (C); and by concurrent stimulation of both ipsilateral and contralateral roots (I + C) to illustrate exact summation of D.R.I, II, III, and IV. Below, full course of dorsal root potentials similarly evoked and similarly identified to illustrate almost complete occlusion of D.R.IV. Bullfrog preparation.

that appears in contralateral recording, or in ipsilateral recording when reactivity of the spinal cord is reduced by asphyxia is closely tied with events in primary afferent fibers. This non-occluding fraction of D.R.IV is most reasonably interpreted as being due to the activity of primary afferent fibers. However the evidence so far presented is compatible with the view that secondary

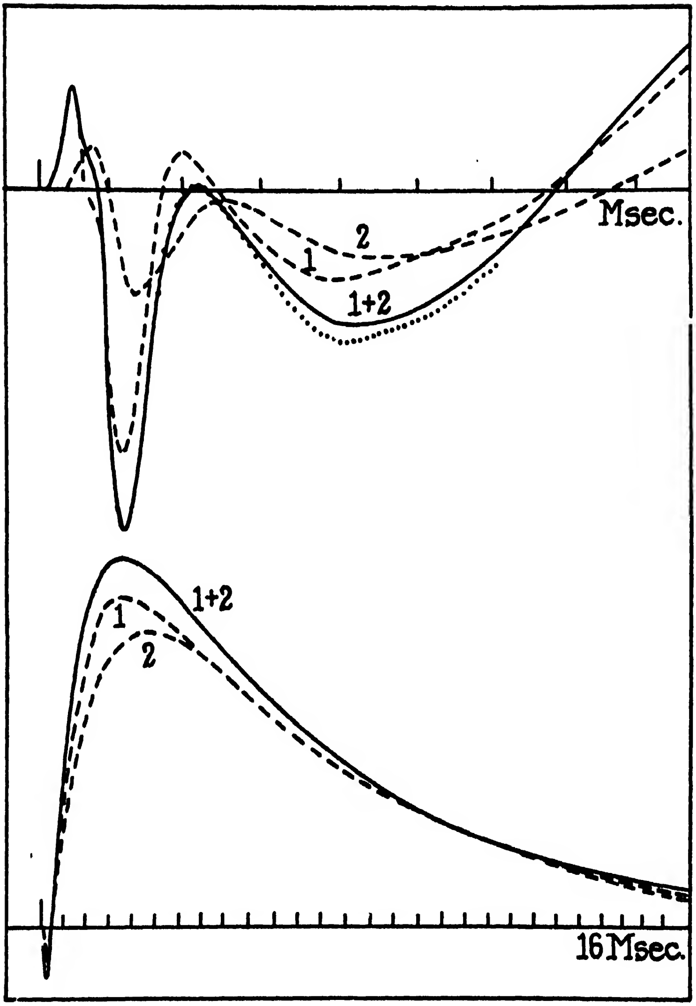


FIG. 9. Above, identified by broken lines, 1 and 2, is seen the early course of dorsal root potentials elicited by stimulation in isolation of two dorsal roots, the recording being from a third, all three roots pertaining to the same side. Identified by the solid line (1 + 2) is the dorsal root potential, similarly recorded, but evoked by concurrent stimulation. The dotted line, constructed by addition of 1 and 2 illustrates the slight occlusion of D.R.IV attending concurrent stimulation. Below, later course of the dorsal root potentials illustrating almost complete occlusion of D.R.V. Bullfrog preparation.

neurons are responsible for the non-occluding fraction of D.R.IV if one makes certain ancillary assumptions: that the responsible group of secondary neurons

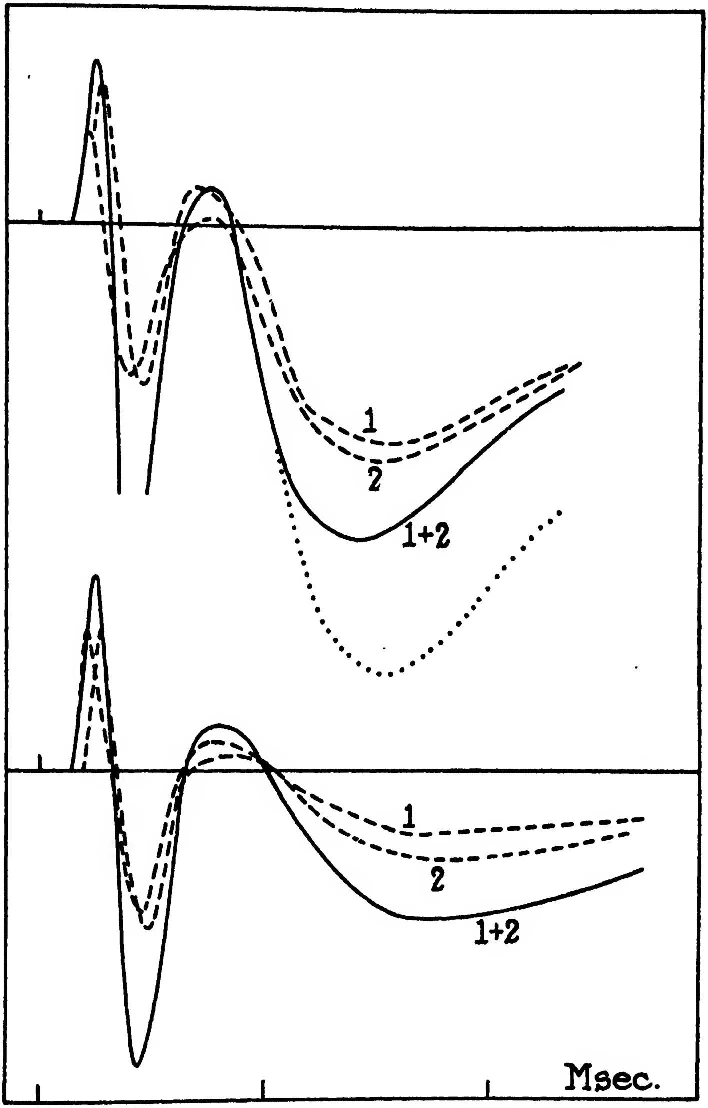


FIG. 10. Experiment similar to that illustrated in Fig. 9, but from a cat preparation. The broken, solid, and dotted lines have the same significance as that given in the legend of Fig. 9. Above, normal preparation, from which it is seen that D.R.IV is occluded to an extent typically greater than is found in experiment with the bullfrog preparation (but not to the same extent as is D.R.V in either preparation). It is significant that occlusion does not appear in the early course of D.R.IV either here or in Fig. 9. Below, as above, but in the partially asphyxiated state, sufficient for the removal of D.R.V. Note absence of occlusion.

would stand in "one-to-one" synaptic relation with primary afferent fibers, that the primary afferent fibers supplying those secondary afferent fibers do so without functional overlap, and that the activity of these secondary neurons does not lead to subsequent production of D.R.V potential. These assumptions being rather unlikely, it is concluded that the non-occluding fraction of D.R.IV results from polarization of primary afferent fibers by the activity of other primary afferent fibers. In the analysis of dorsal root potentials that follows certain consequences of the foregoing conclusion emerge. They may be put to test.

The remaining fraction of the D.R.IV potential, by the very fact that it exhibits occlusion, must be adjudged the result of activity in secondary neurons.[2]

Preliminary Considerations for an Analysis of Dorsal Root Potentials

An analysis of dorsal root potentials in a neighboring root must take into account a sequence of five deflections. The first three of these are *a priori* the result of activity in primary afferent fibers. The fourth apparently is due in part to the activity of primary afferent fibers, and in certain circumstances in part due to the action of secondary neurons. The last potential change of the sequence is, in its measurable extent, surely the activity of secondary neurons. It is profitable at this point to investigate dorsal root potentials in the light of what little is known of the neuronal architecture of the spinal cord and of recent advances in the general understanding of interaction between active and inactive fibers in nervous tissue (19, 24).

Considerations of an Anatomical Nature.—Primary afferent fibers on reaching the root-cord junction penetrate the dorsal columns with an approximately dorsoventral orientation and bifurcate in Y- or T-shaped fashion into longitudinally orientated fibers that ascend and descend the dorsal column. From the parent fibers and from their longitudinal projections, collaterals are directed into the gray substance. Of these collaterals some, derived from the parent fibers of greatest caliber, penetrate to the ventral horn. Others, in dense bundles, flow into the intermediate region. Still others curve on themselves to enter the dorsal horn by a ventral approach. In short the collaterals course through the gray substance in a variety of directions. Since each dorsal rootlet of a group contains a representative population of primary afferent fibers, it follows that the intramedullary projections of neighboring rootlets will lie in parallel array, not only in their longitudinal course within the dorsal column,

[2] The ingenious hypothesis of Barron and Matthews advanced to account for occlusion of D.R.V is relevant only to their assumption that D.R.V represents activity of primary afferent fibers. Once the assumption is seriously questioned, the older view that occlusion is a sign of response to convergent pathways (27) stands until proved incorrect.

but also in the gray substance, regardless of the direction taken therein by the individual collaterals. Now if of two neighboring rootlets one be stimulated and the other not, it further follows that, whatever their course in the cord, active and inactive fibers will lie in parallel. This is a fact of fundamental importance because it makes possible a treatment of dorsal root potentials in terms of relatively simple models of interaction between nerve fibers (24) without the necessity for intimate knowledge, which we do not now possess, of the exact spatial relationships between the many dendritic and axonal structures thrown into activity as a sequel to dorsal root stimulation.

Another important consequence of the parallel arrangement of primary afferent collaterals from neighboring roots concerns the polarization of them by the action of secondary neurons. Stimulation of a dorsal root, as is well known, leads to secondary activity as well as primary activity. Apart from the immediate consequences of impulse conduction by that dorsal root and its intramedullary projections, it follows from the fact of parallel orientation with intramedullary projections of a neighboring root that, whatever the orientation of the secondary neurons with respect to the primary projections, the net polarization of the latter by secondary activity, and so the resulting electrotonus in the two dorsal roots, will have similar duration and electrical sign, and approximately the same intensity.

A special case arises in consideration of dorsal root potentials led from contralateral roots. Primary afferent fibers from the two sides lie in parallel within the dorsal columns, but the collaterals diverge, each group to enter the gray substance of its side. The consequences of this fact emerge in subsequent discussion of ipsilateral and contralateral dorsal root potentials.

Considerations Relating to the Interaction of Neighboring Fibers in a Volume Conductor.—Since primary afferent fibers of a given rootlet may be considered as having similar properties, since they are activated synchronously by a single stimulus, and since the direction of their individual collaterals in the first approximation is inconsequential for the purpose of analyzing dorsal root potentials in a neighboring ipsilateral rootlet, they may be represented by a single fiber (A) of the shape illustrated by diagram A of Fig. 11. Likewise the primary afferent fibers of a neighboring rootlet may be represented by a similar fiber (N). Arrows in fiber A indicate, in diagram A of Fig. 11, the direction of impulse conduction from the extramedullary segment into the volume conductor (stippled) constituted by the spinal cord, thence in three directions, into the longitudinal fibers of the dorsal column and into the collaterals.

It is obvious that the electrical sign of electrotonic potentials in the extramedullary (E) segment of fiber N will depend finally upon the direction of current flow through the membrane of the intramedullary continuation of the parent fiber or I segment, and that this intramedullary continuation of the parent fiber lies in parallel with part of the intramedullary projection of fiber

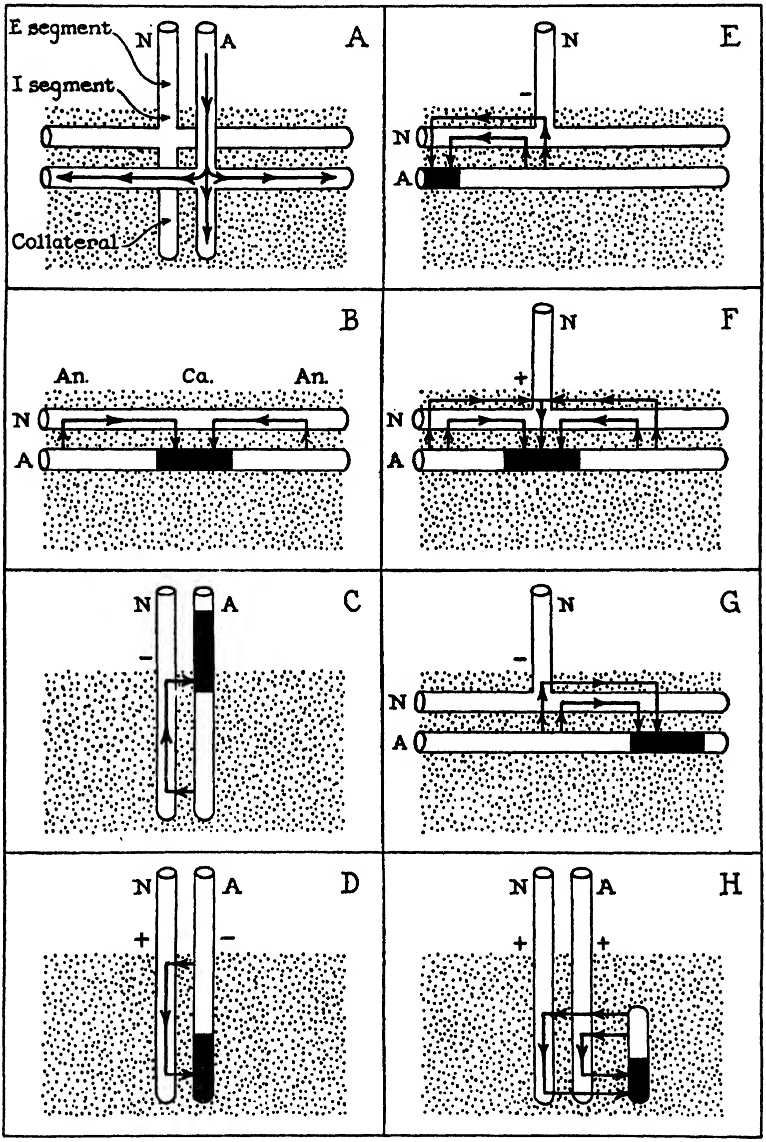


FIG. 11. Fundamental propositions relating to the analysis of dorsal root potentials. Fully described in text. In this figure and in Figs. 12, 14, and 17, fibers A and N represent active and neighboring fibers respectively; the shaded area represents the volume conductor; the black area of fiber A, in each instance, represents the impulse proper; while arrows indicate the external field about the active fibers. Plus and minus signs indicate the direction of electrotonus in the extramedullary (E) segment resulting from physical spread of current polarizing the intramedullary (I) segment.

A and at right angles to the remainder. It is convenient whenever possible to treat as separate problems the interactions resulting from impulse conduction in the parallel and 90° components of fiber A, synthesizing the two only as required by special considerations.

Fig. 11 presents in diagrammatic form the propositions necessary for a qualitative analysis of electrotonus in dorsal roots. Diagram B, recognizable as the fundamental proposition relative to the interaction of parallel neighboring fibers (19, 24), describes the changes that take place in resting longitudinal fibers as a volley of impulses travels along the dorsal column (26). Fiber N forms part of the external conductor of fiber A. Arrows indicate the three phases of membrane current in fiber A: outwards, inwards, outwards. In fiber N membrane current flows in a direction opposite to that in fiber A: inwards, outwards, inwards. The interaction is only detectable by measurement of threshold changes associated in fiber N with the anodal-cathodal-anodal succession of current flow through its membrane. It is important, because the principle is generally applicable, to note that an electrode placed near fibers A and N (*i.e.* on the dorsal column), and pitted against an electrode elsewhere on the preparation records the changes of membrane current in the active fiber A, not those in the neighboring fiber N.

Considered in connection with diagrams C and D of Fig. 11 are the interactions possible as the result of impulse conduction in fiber A from parent fiber to collaterals. Until the crest of the action reaches the root-cord junction current flow in the I segment of fiber N will be outgoing and the electrotonic potential in the E segment will be negative (diagram C). After the crest of the action passes the root-cord junction and until the collateral completes its recovery, current in the I segment will be ingoing and the resulting electrotonic potential of the E segment will be positive (diagram D). The situation described by diagrams C and D of Fig. 11, is none other than a special form of the situation at a fork in nerve (24, 13) and the proposed interaction therefore may be regarded as established in principle.

When an impulse travels in the longitudinal segment of fiber A the pattern of interaction between fiber A and fiber N is more complex, as may be appreciated by reference to diagrams E, F, and G of Fig. 11. To simplify the argument it is assumed for the present that the dorsal roots A and N are sufficiently far apart that interaction between the parent fibers is negligible. Further simplification by omitting the collateral of fiber N is possible since current flow there as the result of conduction in the longitudinal segment of fiber A is not of consequence to the I segment of fiber N. Illustrated by diagrams E, F, and G of Fig. 11 are three stages of interaction as the impulse in the longitudinal segment of fiber A approaches, resides at, and regresses from the level of dorsal root N. Current flow in the longitudinal segment of fiber N of course has the character defined by diagram B of Fig. 11, but the fact that the parent fiber of N enters the conducting medium has important additional consequences.

Briefly stated, membrane current will flow in the I segment of fiber N in a direction identical with that in the longitudinal segment A. Thus while the longitudinal segment of fiber N is polarized anodally by the outgoing current of A, the I segment is polarized cathodally, and the electrotonic potential of the E segment is negative (diagram E). Next in order, as the sink of current flow in the longitudinal segment of fiber A reaches the level of root N, the longitudinal segment of fiber N is cathodally polarized by the inward flowing current about A while the I segment is anodally polarized resulting in anelectrotonus of the E segment (diagram F). Finally, as the impulse in A recedes (diagram G) the entire sequence reverses again causing a catelectrotonic potential to appear in the E segment of fiber N.

In contrast to diagrams B, C, and D of Fig. 11, which are based on theoretical propositions that have already received experimental verification, the predictions of diagrams E, F, and G, as they apply to the I segment, lack prior experimental verification in a system which, by its evident simplicity, permits unequivocal demonstration of their validity. The following section is devoted to experimental confirmation of these latter predictions.

Diagram H of Fig. 11 serves to illustrate the only generally acceptable proposition concerning polarization of primary afferent fibers by the action of secondary neurons. Deliberately to discourage overly facile identification, in diagram H of Fig. 11, of the external polarizing source with any supposed or real structure, it is represented as a simple dipole devoid of anatomical meaning. Diagram H is but one of an infinite number of diagrams that might be drawn to illustrate the fact that, as long as fiber A and fiber N lie in parallel array, the result of polarization by a secondary source must be qualitatively similar in the two fibers.

On the Interaction, in a Volume Conductor, between Parts of Nerve Fibers Lying at Right Angles to One Another.—The theoretical argument advanced in connection with diagrams E, F, and G of Fig. 11 is susceptible of experimental verification in a nerve model. Fig. 12 illustrates the experimental arrangement and the expectation that electrotonus in the insulated segment will pass through three successive stages: cathodal, anodal, and cathodal respectively. It will be noted that the nerve model in many details differs from the spinal cord. For instance the absence of secondary neurons and collaterals removes any possibility that structures other than the longitudinal traject of fiber A could contribute to, or subtract from, the result. Furthermore, in the nerve model fiber N is L-shaped rather than T-shaped, giving rise at the angle to interesting differences in the course of interactions as may be appreciated by comparing Fig. 12, B, C, and D with Fig. 11, E, F, and G, but the qualitative aspects of polarization in the I segment of the primary afferent fibers and in its analogue in the nerve experiment should be similar.

Fig. 13 illustrates the result of an experiment, utilizing a bullfrog sciatic

nerve preparation in the manner defined in diagram A of Fig. 12. An electrode (A) was placed in contact with the "active" nerve near the fork, and at some distance in the volume conductor another electrode (B) was located. Since electrode A is situated at a distance from the point of entry of impulses into the volume conductor, electrodes A-B should record, as a triphasic deflection, the

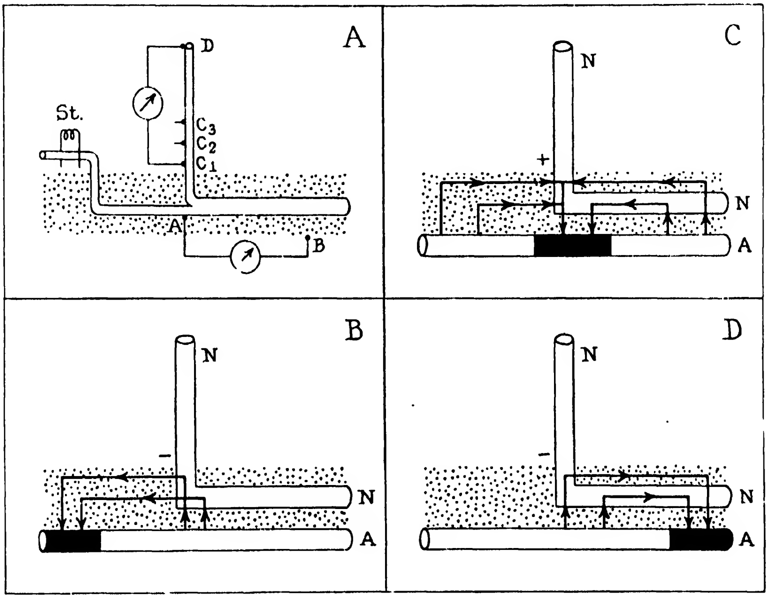


FIG. 12. In diagram A is illustrated the experimental arrangement for demonstration in nerve of interaction between fibers orientated at 90° to each other. Bullfrog sciatic nerve with its two principal subdivisions. St, location of stimulating electrodes. A-B, leading arrangement to record impulses in the active fibers. C-D, leading arrangement to record electrotonus in the inactive fibers, positions C_1, C_2, C_3 of electrode C serving to demonstrate the electrotonic decrement of potential changes in the inactive fibers. Diagrams, B, C, D, to illustrate the anticipated course of interaction as impulses in active fiber A approach, reside at, and depart from the region of 90° orientation.

passage of impulses evoked by stimulation through electrodes St. The "neighboring" branch of the sciatic nerve was drawn up into oil, in such a manner as to leave a short stretch near the fork within the volume conductor to form with the active branch a 90° angle. Electrodes C and D were placed on the insulated segment, the former held by a micromanipulator so that it could assume the successive positions C_1, C_2, C_3 , in order to record electronic propagation into the insulated segment and demonstrate its decremental character.

Record A of Fig. 13, obtained by use of the leading electrodes A-B (Fig. 12),

contains the familiar triphasic deflection expressing changes in membrane current during the passage of a volley of impulses (23). The sequence, as antici-

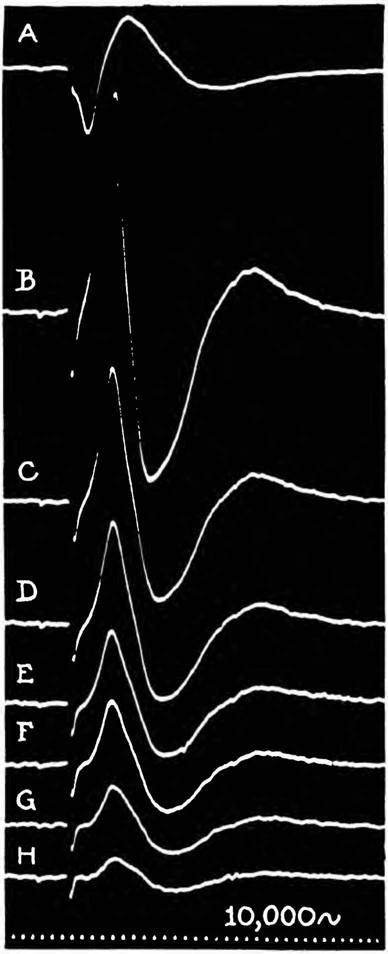


FIG 13. Experimental findings relating to the recording arrangement of Fig. 12 A and in confirmation of the interactions proposed in Fig. 12, B, C, and D. Record A, obtained by means of electrodes A-B in Fig. 12 A. Records B to H, obtained by means of electrodes C-D in Fig. 12 A, the successive records being made with electrode C at locations C_1, C_2, C_3, ...

pated is positive-negative-positive. Illustrated in B to H of Fig. 13 are records obtained by the use of electrodes C-D, the successive observations recording the change as electrode C is moved, in millimeter steps from C_1 to C_2 to C_3.... For observations B to H of Fig. 13 negativity at electrode C relative to elec-

trode D is recorded upwards. It is clear that the electrotonic currents in the insulated segment exhibit in succession an outward, an inward, and an outward direction. In accordance with expectation, test stimulation, in the neighborhood of C_1, of the insulated segment reveals there a succession of enhancement, depression, and enhancement.

One need hardly emphasize the purely qualitative nature of the present analysis of interaction between fibers, undertaken for the sole purpose of verifying the physical possibility of the previously unsubstantiated propositions (Fig. 11, E, F, and G) necessary for analysis of dorsal root potentials.

Analysis and Interpretation of Dorsal Root Potentials

Analysis of D.R.I, II, III.—Since the first three deflections of the dorsal root potential bear a remarkable resemblance to the intramedullary spike potential that signals conduction of a volley within the dorsal columns, there can be little doubt that interaction of the type outlined in diagrams E, F, and G of Fig. 11, by accounting for the electrical sign of these deflections, contains the elements of a satisfactory interpretation of their origin. However, in order to simplify the initial presentation, in Fig. 11, E, F, and G, of the concept of interaction between fibers lying at right angles to one another, a limiting case was chosen, that in which the dorsoventral components of fibers A and N are too far apart for effective interaction. Fully to describe the origin of D.R.I, II, and III it is necessary to present the other limiting case, that in which the dorsoventral components of fibers A and N are so close together that the root-cord junction of fiber N is in effect at the point of entry into the volume conductor of impulses in fiber A. This limiting case, presented diagrammatically in Fig. 14, is particularly interesting, for, despite the fact that the intramedullary spike potential recorded at the root-cord junction of fiber N (and naturally of fiber A) is diphasic, negative-positive (*cf.* reference 23), D.R.I, II, and III are all present in the E segment of fiber N.

For construction of Fig. 14 it will be seen that no propositions have been employed other than those advanced in Fig. 11. When fibers A and N are immediately adjacent (diagram 14 A) it is the entering impulse in the parent fiber A that determines outward flow of current in the I segment, and the appearance of catelectrotonus in the E segment of fiber N. After the crest of the action enters the cord and until the beginning of recovery in the longitudinal segment of fiber A (diagram 14 B) three sources of current flow, S_1, S_2, and S_3, exist in fiber A, all of which are capable of causing current to flow inward through the I segment of fiber N with resulting anelectrotonus in the E segment. Finally, as recovery of the longitudinal segment of fiber A progresses (diagram 14 C), current again will flow outwards in the I segment of fiber N producing catelectrotonus in the E segment.

It is obvious that, in most instances, the recorded D.R.I, II, and III deflections will represent current flows of complex origin intermediate between the

two limiting cases. No useful purpose would be served by carrying this analysis further, since an individual problem is raised by each experimental arrangement for the recording of dorsal root potentials.

Analysis of the Primary Fraction of D.R.IV.—Following a study of the properties of D.R.IV, it was concluded above that a considerable fraction of this deflection, the non-occluding or primary fraction, could most reasonably be attributed to the activity of primary afferent fibers. If this be true, it further

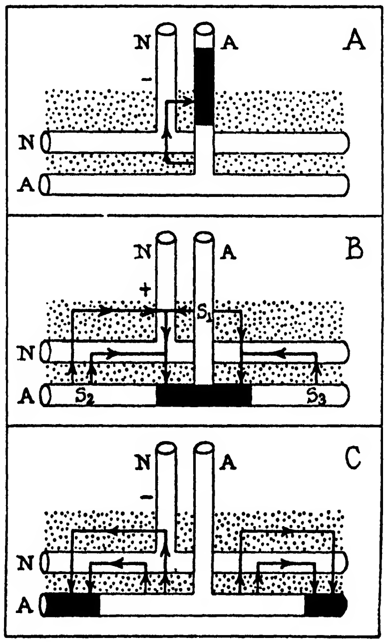


FIG. 14. Diagrams to illustrate one limiting case, the other being illustrated in Fig. 11, E, F, and G, for fiber interaction determining the production of the D.R.I, II, and III deflections of dorsal root potentials.

follows that the responsible activity must be centered in the collaterals, since it is unreasonable to attribute activity having the character of D.R.IV to simple conduction along tract fibers the known properties of which so closely resemble peripheral nerve.

Recourse to the fundamental propositions of Fig. 11, reveals that D.R.IV could only result from the current flows described in diagram D and diagram H. That fraction of D.R.IV that results from activity in primary afferent collaterals (the non-occluding or primary fraction) must arise in the manner of diagram 11 D, the occluding fraction must arise in the manner of diagram 11 H.

Considering the positive sign and prolonged duration of the primary fraction

of D.R.IV, it is possible to predict that this potential deflection represents a persistent negativity in the primary afferent collateral A, resembling rather the residual negativity at a nerve block described by Lorente de Nó (22) although in this instance the gradient must be relatively much more powerful. This prediction carries with it a consequence that may be put to experimental test. Since the parent fibers certainly have recovered during the period of D.R.IV, it would be expected that the supposed residual negativity of the active collaterals should be propagated backwards into the parent fibers and would appear in the active dorsal root as a negative deflection at the more proximal of two recording leads placed thereon.

Now it is known that the dorsal root potential of an active root is a complex event (*cf.* discussion in relation to Fig. 1 A). A negative potential difference of the sort postulated would be written upon the fiber potentials of the root itself and would be overlaid by the large negative potential difference paralleling D.R.V. The required procedure for its demonstration then rests upon the fact that D.R.IV during asphyxia is less labile than D.R.V, and more labile than the potentials relating to spike conduction (including in this instance the after-potentials of the active dorsal root fibers in contact with the recording leads). Polarization potentials, the result of the stimulating current, should not change by virtue of intramedullary changes during asphyxia. With these propositions in mind, one may consider the observations recorded in Fig. 15, taken from the same experiment as was Fig. 6, but showing changes wrought by asphyxia in the active rather than in a neighboring root.

The records reproduced in Fig. 15 were made during the course of recovery from a period of asphyxia. Figure 15 A illustrates the course of dorsal root potential that was maintained constant over a period of some minutes, and thus is taken to be the contribution from extramedullary sources. Following re-establishment of respiration (and circulation[3]) the dorsal root potential changed first to the form illustrated in Fig. 15 B, and later recovered through the stages represented by records C to G. Fig. 15 clearly indicates that, in addition to the extramedullary components of the dorsal root potential in an active root, there are two gradients of negativity, exhibiting differing temporal course and differing susceptibility to asphyxia. The more resistant of these, as judged by earlier recovery after asphyxia, is represented by the difference in potential level between records A and B of Fig. 15. When plotted on an isopotential base line (dotted line in Fig. 15) the potential difference represented by (B-A) is seen to match closely, but with opposite electrical sign, the D.R.IV deflection recorded in similar conditions from a neighboring root (Fig. 6E).

Identification of the early negative potential difference in an active root with

[3] In the course of the experiment from which Figs. 6, 15, and 18 were prepared in each instance the asphyxia was so severe that cardiac arrest resulted. Circulation was restored by massaging the chest.

the positive D.R.IV of a neighboring root is greatly facilitated by means of simultaneous recordings from the two roots in question. Fig. 16 presents an experiment in which asphyxial changes in the dorsal root potential of the active root (A to D above) and of a neighboring root (A to D below) were recorded simultaneously with the aid of a twin-beam oscillograph. In each instance A, B, C, and D illustrate successive changes in the dorsal root potentials from the onset of asphyxia (A) until only D.R.I and D.R.II remained in the dorsal root potential of the neighboring root (D). Records A, B, and C illustrate the

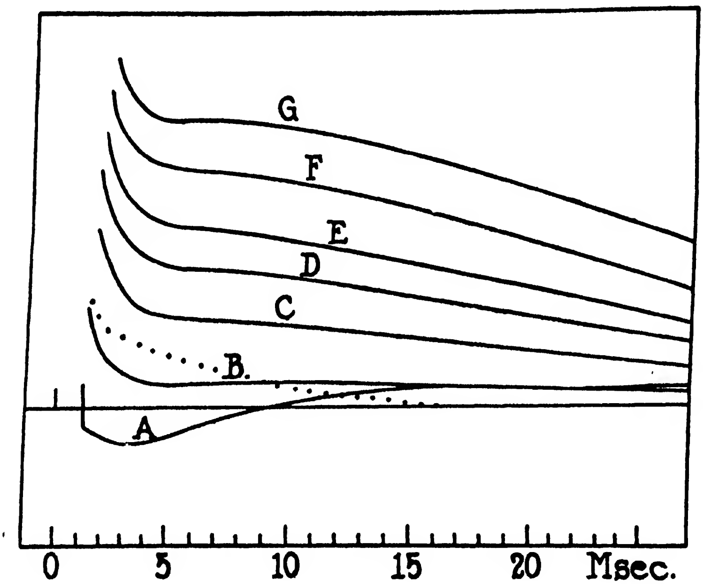


FIG. 15. Modification by asphyxia of dorsal root potentials recorded from a stimulated root. A, course of dorsal root potential after prolonged asphyxia. B, first stage of recovery. C to G, subsequent stages of recovery to normal. The dotted line plots on an isoelectric baseline the potential difference between records A and B.

progressive loss of D.R.V and its homologue in the active root. The further change (D) in the active root, consisting of loss of the early negative potential, exactly parallels the loss of D.R.IV in the neighboring root. For this reason it is concluded that the two potential changes indeed are associated. This being so it is a necessary consequence of their electrical signs that the causal activity resides not in secondary neurons, but in the active primary afferent fibers.

Concerning Potentials in the Terminal Regions of Presynaptic Fibers.—The foregoing observations call for the existence, following the arrival of impulses at the primary afferent endings, of an enduring flow of current in the direction from parent fibers to terminal regions. In the absence of interfering secondary activ-

ity such a current flow would be recorded by a microelectrode appropriately situated in a nucleus of termination as a negative wave detectable for nearly 15 msec. and decaying over an approximately exponential course to half-value

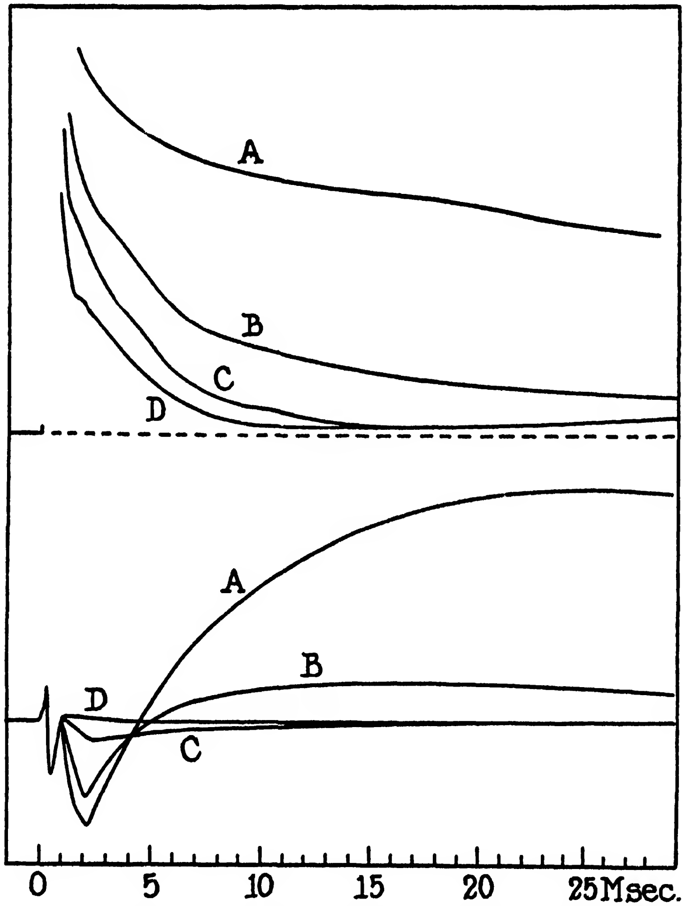


FIG. 16. Simultaneous recording of dorsal root potentials from the stimulated root (above) and a neighboring root (below) to show, in A, B, and C parallel progress in the two roots of asphyxial block of the D.R.V deflection and its homologue, but in particular to illustrate, by comparison of the potential level of C and D in each case, the parallel loss of early negativity in the active root and of D.R.IV in the neighboring root.

in slightly less than 3 msec. In experiments that fulfill the stated conditions, Brooks and Eccles (7) have recorded such a potential to which they have given the name "focal synaptic potential." It is their hypothesis that the sinks of

current flow giving rise to this recorded potential difference lie in the motoneuron somata, the sources at the motoneuron axons, whereas the present experiments demand that a similar if not identical potential difference must be recorded by virtue of sinks of current flow located in the presynaptic fibers at or close to the endings, the sources being located in the presynaptic fibers at some greater distance from the endings.

Potentials referable to activity in the terminal regions of presynaptic fibers have been recorded, but not free from interference by secondary activity, in the oculomotor nucleus by Lorente de Nó (22) and in the quadriceps nucleus of the spinal cord by Renshaw (25). While postulating that the "β deflection" of Renshaw is identical with their "focal synaptic potential" Brooks and Eccles (7) have disputed Renshaw's conclusion that his "β potential" is produced by the terminal portions of the presynaptic fibers. Considering the present evidence, however, it would seem that Renshaw's surmise undoubedly was correct.

It should be emphasized that the recording by microelectrode within a motor nucleus of a potential change of the type under discussion does not yield of itself sufficient information by means of which to decide whether the recorded potential is referable to presynaptic or postsynaptic structures. Since the potential change in the nucleus is of negative sign it follows that the sinks of current flow are there. It likewise follows, in the monosynaptic system, that the sources must be either in the motoneurons, or in the primary afferent fibers. On the assumption that the motor axons supply current to the somata during the "synaptic potential," Brooks and Eccles (7) regard the existence of a ventral root "synaptic potential" as proof of the postsynaptic origin of the "focal synaptic potential." But the sources of current flow that determine the course of the ventral root synaptic potential outlast by two to three times the sinks that determine the "focal synaptic potential" (7). On the contrary the sources in primary afferent fibers that determine the appearance in an active dorsal root of the early negativity (Figs. 15 and 16) have a duration comparable to that of the sinks determining the "focal synaptic potential." In view of this last fact it seems unlikely that electrotonic slowing assumed to occur in the intramedullary course of the ventral root fibers (14) could account for the discrepancy between "focal synaptic potential" and ventral root "synaptic potential." In the circumstances the simplest conclusion must be that the two "synaptic potentials" are manifestations of different systems of current flow. One may assume a causal relationship while recognizing the obscurity of its nature.

It is important to recognize that the fraction of dorsal root potential of primary origin (as seen in Fig. 7 B) cannot reflect in precise detail all phases of the potential sequence in the terminal regions of the presynaptic fibers although in general they are not dissimilar except in electrical sign. To simplify the argument the reasons for this fact are presented diagrammatically in Fig. 17. From a study of diagrams A, B, and C of Fig. 17 it will be seen that from the time that

the crest of the action in fiber A reaches the volume conductor of the spinal cord (immediately after the condition represented by diagram A) until it reaches the presynaptic terminals (diagram C) the polarization of the I segment of fiber N, and consequently the dorsal root electrotonus, is caused by a flow of current different from that surrounding the collaterals (diagram B). Consequently it is only after the crest of the action reaches the presynaptic ter-

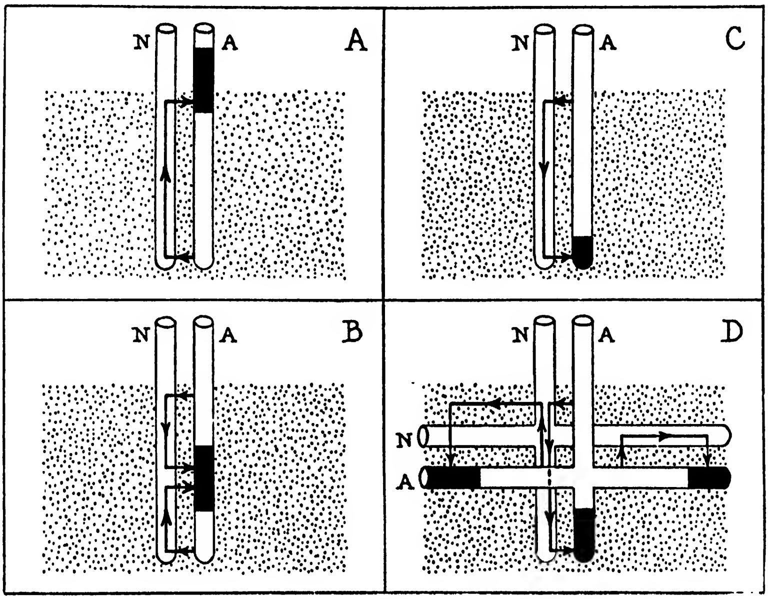


FIG. 17. Diagrams to illustrate the fact that D.R.IV and the potential changes of active primary afferent collaterals for part of their time course could be related to the same current flows, but that also for part of their time course they cannot be so related, the dorsal root electrotonus being determined by a current flow different from that surrounding the collaterals (diagram B). Diagram D illustrates the manner in which the I segment may be subjected to opposing current flows during the course of the D.R.III deflection.

minals (if, indeed, it does) that D.R.IV and an "ending potential" could be compared in detail.

Another factor that must contribute to differences of the sort under discussion may be mentioned in connection with diagram D of Fig. 17, for it provides in addition an understanding of the fact that D.R.III uniformly has not been of as great amplitude as one might reasonably have expected. Diagram 17 D shows how, once the primary impulse conducts beyond the parent fiber, and until recovery following the impulses that continue in the longitudinal segment of fiber A is complete, the I segment of fiber N is subjected simultaneously to

anodal and cathodal flows of current. In short D.R.III is written, not upon an isoelectric base line, but interrupts, so to speak, an otherwise more or less continuous anelectrotonus consisting of D.R.II and D.R.IV.

The Occluding or Secondary Fraction of D.R.IV.—Although there can be no reasonable doubt but that the occluding fraction of D.R.IV results from the activity of secondary neurons, there is no direct evidence bearing on the mode of its production. Indirect evidence, however, would seem to justify the view that the occluding fraction is produced by essentially the same type of activity on the part of the secondary neurons as that which in primary afferent fibers are responsible for the appearance of the non-occluding or primary fraction. The argument is as follows: Activity in primary afferent fibers, which is entirely axonal, produces a D.R.IV potential, but no D.R.V potential. Secondary activity, in which, among the internuncial pools, both axons and somata are equally involved, produces both a D.R.IV potential and a D.R.V potential. Secondary activity elicited by antidromic volleys, in which case the activity in the main must be referred to somata, produces no D.R.IV potential but (in the bullfrog) results in a dorsal root potential indistinguishable from D.R.V. Finally, given a parallel orientation of secondary axons and primary afferent fibers (as is found, for instance, between the axons of neurons of the intermediate nucleus and the "reflexomotor" collaterals) the essential anatomical requirement is satisfied, and activity of secondary collaterals becomes a sufficient explanation of the occluding fraction of D.R.IV.

It has not been possible in a satisfactory manner to distinguish among the components of the dorsal root potential in an active rootlet one that corresponds to the occluding fraction of D.R.IV in a neighboring rootlet.

Ipsilateral and Contralateral Dorsal Root Potentials.—Dorsal root potentials recorded in an adjacent ipsilateral root and in a contralateral root differ in two important respects: (*a*) The secondary fraction of D.R.IV is relatively large in ipsilateral recording, relatively small or absent in contralateral recording, and (*b*) a D.R.V deflection appears contralaterally only after considerable latency and at a time when ipsilateral D.R.V may have reached 60 to 70 per cent of peak amplitude. Both of these differences express the fact that secondary activity centered in the gray substance of one-half of the spinal cord does not in a significant degree polarize primary afferent fibers of the other side. There could be two reasons for the fact, both anatomical. It may be, at the distances involved, that the field generated by active secondary neurons is too weak to cause appreciable effect, or it may be that the orientation of the field is such that little or no net polarization of contralateral primary afferent fibers results. The two factors are not in any way mutually exclusive.

Since the primary afferent collaterals on the two sides of the cord diverge and course laterally to splay out into the gray substance, it is improbable that activity confined to the endings of one group could influence the other group any

more than could secondary neurons of the contralateral gray substance. The fact that a D.R.IV deflection is recorded contralaterally therefore can only be explained by the additional fact, demonstrated in Figs. 15 and 16, that the residual negativity of primary afferent collaterals propagates itself backward into the I segment (and hence presumably also into the longitudinal fibers) which parts of the primary neurons are suitably orientated in sufficient proximity (as shown by the appearance of D.R.I, II, and III in contralateral recording) to permit the necessary interaction to take place.

The appearance of a D.R.V deflection in contralateral recording apparently depends upon the spread, by decussation through commissural neurons, of activity from one to the other half of the spinal cord.

On the D.R.V Deflection.—It has already been stated that the present experiments add little to what is known of the D.R.V deflection. Since this prolonged negative wave appears in active and neighboring roots alike with the same electrical sign, it follows from the propositions considered in connection with Fig. 11 that secondary neurons are responsible for the polarization that produces this deflection. To this extent the present experiments are in agreement with the interpretations of Bonnet and Bremer (2, 3) and of Eccles and Malcolm (16). Furthermore, for reasons that have been discussed, it seems likely that the activity of somata rather than that of axons is the causal agent.

The observation at times has been made, initially by Barron and Matthews in their original description of "the dorsal root potential" (1), that the D.R.V deflection evoked by a dorsal root volley parallels in time course the positive intermediary potential (17) similarly evoked. It would seem that this is a significant fact, and, as a first approximation, it would further seem justified to assume as did Barron and Matthews, that the two phenomena are intimately related despite the obscurity that at present surrounds the relationship.

D.R.IV and Negative Cord Potential.—During the analysis of the D.R.I, II, III complex it was seen that these deflections bear a reasonably definable relation to the intramedullary spike potential of the cord potential. Likewise there exists a close similarity, but ill defined relationship between D.R.V and the positive intermediary potential. The similarity between D.R.IV and negative intermediary potential at first sight, however, is neither close nor well defined. Some observations on cord potentials during the course of asphyxia have interest in this connection. Illustrated in Fig. 18 are records, superimposed by tracing, of cord potentials recorded at various stages of asphyxia by means of electrodes placed one upon the dorsum of the cord, the other at a distance on non-nervous tissue. The records are from the same preparation as that from which Figs. 6 and 15 were obtained. Record A of Fig. 18 represents the normal cord potential, B to E, successive stages in the asphyxial effect. From these recordings it is quite clear that the negative intermediary potential is divisible into two fractions, one associated with and succeeded by positive intermediary

potential, the other not. The latter, seen in isolation in record E of Fig. 18, has properties similar to those of the primary fraction of D.R.IV and the early negativity in an active dorsal root, including virtually identical duration and regression: it would appear to share with them a common origin.

The problem raised by the electrical sign of the presumably primary fraction of the negative intermediary potential (Fig. 18 E) is different from that involved in the study of similar deflections in dorsal roots for the precise orientation of the individual collaterals within the volume conductor of the spinal cord

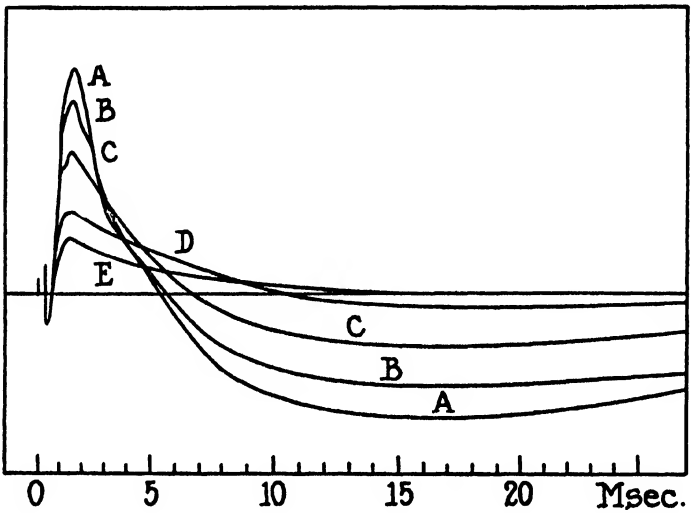


FIG. 18. Cord potentials recorded with one electrode on the cord dorsum, the other on non-neural tissue, to illustrate progressive stages, A to E, in the course of modification by asphyxia. The negative intermediary potential contains two components separable by asphyxia, one of which (E) in many ways is comparable with D.R.IV.

can no longer be neglected. In other words a unique solution is only possible following a study of the fields about active primary afferent fibers in the light of knowledge as to the precise orientation of the active fibers. The knowledge not being available the problem cannot as yet be solved.

The other fraction of negative intermediary potential is clearly the result of internuncial activity (17), possibly on the part of the interneurons responsible for the secondary fraction of D.R.IV. There is not much resemblance between these potentials, a fact that could mean among other things merely that the aspect of internuncial activity that dominates the intermediary potential does not possess the appropriate spatial orientation to result in a net polarization, one way or the other, of the I segment of primary afferent fibers. Since the action of secondary axons is the likely cause of the polarization producing the secondary fraction of D.R.IV, one might suppose that activity in inter-

nuncial somata predominates in the cord lead, and so accommodate the discrepancy.

On the Relative Susceptibility to Asphyxia of Various Parts of the Reflex Arc.— The present experiments utilizing asphyxia have provided an opportunity to study the relative sensitivity to asphyxia of the several structures encountered by impulses on their way through the spinal cord. To recapitulate the experimental observations, in each instance D.R.V and the occluding (secondary) fraction of D.R.IV disappear together, leaving for a short period the D.R.I, II, III deflections and non-occluding fraction of D.R.IV (Figs. 7 and 10). Next to fail with continuing asphyxia are the remainder of D.R.IV and D.R.III. A resistance to asphyxia comparable to that of peripheral nerve characterizes the D.R.I and D.R.II deflections. These observations can only mean that the ability of somata to generate impulses in response to presynaptic volleys is the first asphyxial failure, although to judge from the observations of Brooks and Eccles (8) the somata (of motoneurons) should still be able to respond to antidromic impulses. Subsequent and conjoint failure of D.R.III and the non-occluding fraction of D.R.IV indicates that the "weak point" of the primary afferent fibers is the region where parent fibers branch to form longitudinal fibers and collaterals, rather than the collaterals or terminals themselves.

An Analysis of Synaptic Excitatory Action

It has been shown (14, 20) that the facilitatory action of a near synchronous presynaptic volley in a monosynaptic reflex system can be detected for nearly 15 msec., the decay of facilitation being an approximately exponential regression to half-value in 2.8 msec. (20). At the time that the prolonged facilitation was documented reason was given (20) for supposing it to be the expression of a process additional rather than alternative to the brief excitatory action of earlier descriptions. Hence, and because of certain analogies with phenomena at a block in nerve (22), the prolonged facilitation was called *residual facilitation*. In other words it was concluded that presynaptic impulses exert upon postsynaptic neurons two actions: the initial (or detonator[4]) action and a

[4] Attention must be brought to the fact that the term employed herein, and previously (20), is detonator *action*, not detonator *response*, since, to the extent that one term has been substituted for the other, the earlier paper (20) has been misquoted (15). By detonator action is implied, in accordance with Eccles' first use of the term (reference 12, page 6), a brief excitatory action exerted by presynaptic impulses. The term detonator response, again in accordance with original usage (reference 12, page 11), implies an action *sui generis* on the part of the postsynaptic neuron presumed to mediate between the excitatory action of presynaptic impulses and the generation of postsynaptic impulses. If, however, the term detonator action is to be construed in such a way (*cf.* reference 12, page 17) that it includes a detonator response, then present use (20, and above) has been in error.

residual action. Despite differences in detail, this conclusion in itself is nothing more than a reiteration of the views advanced by Bremer (4). From the foregoing experiments and consideration it is now concluded that the residual action of presynaptic impulses consists of a current flow about presynaptic collaterals during the period of residual negativity, and that such current flow about primary afferent collaterals is a sufficient agent to account for the phenomenon of residual facilitation in monosynaptic reflex pathways.

None of the experimental material upon which this paper is based bears on the nature of the event (initial excitatory action) by means of which the transmission of impulses is secured. As a matter of fact two important properties of the initial action, its brevity[5] and sharp spatial decrement (22), preclude the possibility of electrotonic propagation into dorsal roots. However, there is still no reason to doubt the existence of an initial action to which the residual action is appended, and indeed the most recent experiments of Brooks and Eccles (10) have yielded facilitation curves that indicate a brief early phase preceding or superimposed upon the residual facilitation. Furthermore, the conditions that Brooks and Eccles have found necessary for demonstration of the initial facilitation are in full accord with the notion of sharp spatial decrement. Although Brooks and Eccles now accept the postulation of two phases of facilitation in monosynaptic reflex arcs, their interpretation as to origins is necessarily quite different from that here presented, being founded upon the fundamental assumption (7–10, 12–14) that current flow at the presynaptic endings has essentially the brief duration of axon spikes in peripheral myelinated fibers. As a consequence (and in consideration of "synaptic potentials") it was necessary further to assume that the more prolonged phenomenon, by whatever name it may be called, resides in the postsynaptic neurons. Now that the assumption concerning brevity of the presynaptic action is demonstrably untenable, the hypotheses of excitation (10, 13), inhibition (9), and of the origin of the dorsal root potential (16) predicated upon it appear unnecessarily complicated.

Although the major emphasis is now placed upon presynaptic events as agents for transmission and facilitation (not to speak of direct inhibition) it must not be supposed thereby that membrane changes do not occur also in postsynaptic neurons when acted upon by presynaptic impulses. However, in the presence of active presynaptic changes during orthodromic reflex activity, it seems highly unlikely that membrane changes of postsynaptic origin would be revealed by recording at the region of synapsis unless and until the postsynaptic neurons respond in turn with conducted impulses. In the absence of discharge, therefore, the demonstration of threshold change, as by the use of monosynaptic test

[5] To avoid misunderstanding it is well to note that brevity is a factor only in the active root.

reflexes, provides the only clue to the existence of membrane changes at the regions the threshold of which is tested.

The foregoing argument is based upon certain considerations of interaction between nerve fibers. If of two closely proximate groups of nerve fibers in a common conducting medium one group is active in the sense of conducting a volley of impulses, the other not, then: (*a*) fluctuations of membrane current occur in both groups of fibers (24); (*b*) threshold changes associated with the fluctuations of membrane current in the inactive fibers may be detected by means of suitable experimental procedure (24); but (*c*) an electrode placed in close proximity to the two groups of fibers (in association with an electrode elsewhere) records the membrane changes of the active fibers to the effective exclusion, at least, of those in the inactive fibers, unless and until the inactive fibers, by one means or another, as in "ephaptic" phenomena, themselves become active.

SUMMARY

The "dorsal root potential" consists of five successive deflections designated for convenience, D.R.I, II, III, IV, and V. Of these, D.R.V alone constitutes the dorsal root potential of prior description. A study has been made of the general properties of those deflections not previously described.

Dorsal root potentials are electrotonic extensions into the extramedullary root segment, the result of electrical interactions within the cord comparable to those that have been studied in peripheral nerve. Although the anatomical and electrical conditions of interaction are infinitely more complex in the cord than in nerve, it is seen that the fact of parallel distribution of primary afferent fibers pertaining to neighboring dorsal roots provides a sufficient anatomical basis for qualitative analysis in the first approximation of dorsal root potentials.

An extension of the theory of interaction between neighboring nerve fibers has been made to include an especial case of interaction between fibers orientated at right angles to one another. The predictions have been tested in a nerve model and found correct. Given this elaboration, and the stated anatomical propositions, existing knowledge of interaction provides an adequate theoretical basis for an elementary understanding of dorsal root potentials.

The study of general properties and the analysis of dorsal root potentials have led to the formulation of certain conclusions that follow.

D.R.I, II, and III record the electrotonic spread of polarization resulting from the external field of impulses conducted in the intramedullary segment and longitudinal trajects of primary afferent fibers.

D.R.IV arises in part as the result of activity in primary afferent fibers, and in part as the result of activity in secondary neurons. In either case the mode of production is the same, and the responsible agent is residual negativity in

the active collaterals, or, more precisely, the external field of current flow about the collaterals during the period of residual negativity.

Current flow about active primary afferent collaterals during the period of *residual negativity is the agent for residual facilitation of monosynaptic reflex pathways. Since the changes in reflex threshold follow the course of residual negativity there is no need to postulate especial properties for prolonging action at regions the threshold of which is measured by means of monosynaptic test reflexes.*

D.R.V results from polarization of primary afferent fibers by current flow about secondary neurons. There is indication that somata rather than axons of secondary neurons generate the polarizing currents. Similarity between D.R.V and the positive intermediary potential further indicates that soma gradients established during the recovery cycle are responsible for D.R.V.

Little or no net polarization of primary afferent fibers results from activity confined to the contralateral gray substance, the dorsal root potentials in contralateral recording resulting from interaction in the dorsal column or in the ipsilateral gray substance following decussation of contralaterally evoked activity.

During the course of asphyxia the initial defect in reflex pathways is the failure of secondary neurons to respond to primary impulses. Subsequently block is established at the branching zone of primary afferent fibers.

A relation exists between the sequence of dorsal root potentials and the cord potential sequence, the major departure from exact correspondence occurring in the region of D.R.IV and the negative intermediary potential and being of a nature to suggest that different aspects of internuncial activity are emphasized by the two methods of leading.

BIBLIOGRAPHY

1. Barron, D. H., and Matthews, B. H. C., The interpretation of potential changes in the spinal cord, *J. Physiol.*, 1938, **92**, 276.
2. Bonnet, V., and Bremer, F., Etudes des potentiels électriques de la moelle épinière faisant suite chez la grenouille spinale a une ou deux volées d'influx centripètes, *Compt. rend. Soc. biol.*, 1938, **127**, 806.
3. Bonnet, V., and Bremer, F., Relation des potentiels reactionnels spinaux avec les processus d'inhibition et de la sommation centrale, *Compt. rend. Soc. biol.*, 1938, **127**, 812.
4. Bremer, F., Dualité des processus d'excitation centrale, *Ann. physiol. physicochim. biol.*, 1933, **9**, 897.
5. Bremer, F., and Bonnet, V., Contributions a l'étude de la physiologie générale des centres nerveux. II. L'inhibition refléxe, *Arch. internat. physiol.*, 1942, **52**, 153.
6. Bremer, F., Bonnet, V., and Moldaver, J., Contributions a l'étude de la physiologie générale des centres nerveux. I. La sommation centrale, *Compt. rend. Soc. biol.*, 1942, **52**, 1.
7. Brooks, C. McC., and Eccles, J. C., Electric investigation of the monosynaptic pathway through the spinal cord, *J. Neurophysiol.*, 1947, **10**, 251.

8. Brooks, C. McC., and Eccles, J. C., A study of the effects of anaesthesia and asphyxia on the monosynaptic pathway through the spinal cord, *J. Neurophysiol.*, 1947, **10,** 349.
9. Brooks, C. McC., and Eccles, J. C., An electrical hypothesis of central inhibition, *Nature*, 1947, **159,** 760.
10. Brooks, C. McC., and Eccles, J. C., An analysis of synaptic excitatory action, *J. Neurophysiol.*, 1948, **11,** 365.
11. Dun, F. T., and Feng, T. P., A note on two components of the dorsal root potential, *J. Neurcphysiol.*, 1944, **7,** 327.
12. Eccles, J. C., The discharge of impulses from ganglion cells, *J. Physiol.*, 1937, **91,** 1.
13. Eccles, J. C . An electrical hypothesis of synaptic and neuromuscular transmission, *Ann. New York Acad. Sc.*, 1946, **47,** 429.
14. Eccles, J. C., Synaptic potentials of motoneurones, *J. Neurophysiol.*, 1946, **9,** 87.
15. Eccles, J. C., Conduction and synaptic transmission in the nervous system, in Annual Review of Physiology, (V. E. Hall, J. M. Crismon, and A. C. Giese, editors), Stanford, Annual Reviews, Inc., and American Physiological Society, 1948, **10,** 93.
16. Eccles, J. C., and Malcolm, J. L., Dorsal root potentials of the spinal cord, *J. Neurophysiol.*, 1946, **9,** 139.
17. Gasser, H. S., and Graham, H. T., Potentials produced in the spinal cord by stimulation of dorsal roots, *Am. J. Physiol.*, 1933, **103,** 303.
18. Gotch, F., and Horsley, V., On the mammalian nervous system, its functions, and their localization determined by an electrical method, *Phil. Tr. Roy. Soc. London, Series B*, 1891, **182,** 267.
19. Katz, B., and Schmitt, O. H., Electric interaction between two adjacent nerve fibres, *J. Physiol.*, 1940, **97,** 471.
20. Lloyd, D. P. C., Facilitation and inhibition of spinal motoneurons, *J. Neurophysiol.*, 1946, **9,** 421.
21. Lloyd, D. P. C., and McIntyre, A. K., Potentials of dorsal roots and related phenomena, *Fed. Proc.*, 1948, **7,** 74.
22. Lorente de Nó, R., Transmission of impulses through cranial motor nuclei, *J. Neurophysiol.*, 1939, **2,** 402.
23. Lorente de Nó, R., A study of nerve physiology, *Studies from The Rockefeller Institute for Medical Research*, 1947, **131, 132.**
24. Marrazzi, A. S., and Lorente de Nó, R., Interaction of neighboring fibres in myelinated nerve, *J. Neurophysiol.*, 1944, **7,** 83.
25. Renshaw, B., Observations on interaction of nerve impulses in the grey matter and on the nature of central inhibition, *Am. J. Physiol.*, 1946, **146,** 443.
26. Renshaw, B., and Therman, P. O., Excitation of intraspinal mammalian axons by nerve impulses in adjacent axons, *Am. J. Physiol.*, 1941, **133,** 96.
27. Sherrington, C. S., Some functional problems attaching to convergence. Ferrier Lecture, *Proc. Roy. Soc. London, Series B*, 1929, **105,** 332.
28. Woolsey, C. N., and Larrabee, M. G., Potential changes and prolonged reflex facilitation following stimulation of dorsal spinal roots, *Am. J. Physiol.*, 1940, **129,** 205.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, May 1, 1949,
Vol. 89, No. 5, pp. 529–539]

NUTRITION OF THE HOST AND NATURAL RESISTANCE TO INFECTION

IV. THE CAPABILITY OF THE DOUBLE STRAIN INOCULATION TEST TO REVEAL GENETICALLY DETERMINED DIFFERENCES IN NATURAL RESISTANCE TO INFECTION

BY HOWARD A. SCHNEIDER, PH.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, January 26, 1949)

Among students of infectious disease the term "natural resistance" has come to include those attributes of a host, preexistent to the actual event of infection, which tend to lessen the consequences of that infection. It is an obvious thesis that an analysis of these mitigating host attributes might lead to possibilities of their enhancement to the happy end (from the host's view) that the effects of infection might thus be reduced from the serious or fatal to the trivial or inapparent. From a theoretical viewpoint these host attributes may be due to (*a*) genetic or (*b*) environmental causes. The diet a host consumes is one such environmental factor of possible importance and in investigations previously reported (1–3) an analysis has been undertaken of the biological circumstances in which the nutrition of the host is capable of favorably influencing the frequency of survivorship following an initial infection. The disease model employed in these studies was mouse salmonellosis and from the findings it was concluded that host nutritional influences were operative in a genetic framework. This genetic framework was constructed of the various genotypes within the host and pathogen species as they joined in various combinations in the infection experience (see reference 2). In each of these unique collisions of a certain pathogen genotype with a certain host genotype the effect of host nutrition on survivorship frequency had been examined but in this examination a choice was made as to the kind of nutritional difference which was tested. This choice was, in a sense, a limitation on any statements which might be made on the effects of nutrition in general on these events, for the list of nutritive entities which might be manipulated, either singly or in combinations, is almost endless. But in order to begin somewhere, and with the most encouraging prospect, recourse was had to the historical precedent in the development of the science of nutrition. The nutritional difference which was chosen for testing for its ability to bring about differences in survivorship frequency following infection was the nutritional gap existing between a so called "synthetic" diet and a diet of natural foodstuffs.

When the effect of nutrition had been tested in this manner the greatest

increment of increased survivorship on the natural diet was observed when genetically heterogeneous hosts were infected with a pathogen population which in itself was heterogeneous in its genotypic composition with respect to virulence. Further examination of this rôle of pathogen heterogeneity in the nutrition effect led to a change in technique in the experiments designed to permit the concentration and isolation of the factor(s) in the natural diet responsible for the increased frequency of survivorship. This change in technique exploited the concept of the heterogeneous pathogen by employing two different cultures of *Salmonella typhimurium*, one avirulent and the other virulent, and further, by injecting into the host the avirulent culture 24 hours before the virulent one. This procedure has been named the double strain inoculation test, or DSI test. The steps which led to this infection procedure have been described previously (3), but it is worth emphasis that this selection of a 24 hour time interval between the two components of the heterogeneous pathogen population bears only a special relevance for these two particular cultures and this relevance rests solely on the fact that it is at precisely this time interval that the dietary effect is maximal (see reference 3). For other cultures other time intervals, including the zero time interval (see reference 2), might well prove to be maximal in their effect.

Whatever may be the specific details which the DSI test might take in any specific infection the fact remains that its important element of conducting an infection test of "natural resistance" by means of two separate cultures was arrived at by an analysis of the effect of an environmental factor; *i.e.*, diet. The question now arises whether this test can detect differences in "natural resistance" in those instances in which these host attributes are determined not by environment, but by genetics. For there is no evidence, *a priori*, which would assure that differences in "natural resistance" arranged by diet are necessarily the same as differences arranged by genetic composition. Indeed it has been found necessary to devise a special test to detect these diet effects with reliability. What we need to know is whether the DSI test is capable of detecting these genetically arranged differences in survivorship as well as the nutritionally arranged event, or whether it is incapable of so doing and we are thereby reduced to considering the DSI method as dealing fundamentally with something different from the genetically controlled situation. To find an applicability of the DSI method in both the genetic and the nutritional event would have certain advantageous consequences, not the least of which is that it would make possible the beginnings of a unified biochemical theory which would underlie both the nutritional and genetic factors in their separate workings.

It is the substance of this paper that the DSI method is capable of revealing differences in "natural resistance" which are referable to genetic constitution in addition to its established ability to reveal differences in "natural resistance" due to nutrition.

Materials and Methods

Animals.—In the following experiments use has been made of four pure, brother-sister inbred strains of laboratory mice (18th to 29th generation) developed in this laboratory by the late Leslie T. Webster from a common stock of Rockefeller Institute mice and now differing widely in their response to injection. These stocks are maintained free of *Salmonella* infection. The derivation of these strains has been described by Webster (4, 5). Briefly, they are characterized by the four possible combinations of relative resistance and susceptibility to a bacterial disease (mouse typhoid, salmonellosis), and to a virus disease (St. Louis encephalitis); *viz.*, bacteria-resistant, virus-resistant; bacteria-susceptible, virus-resistant; bacteria-resistant, virus-susceptible; and bacteria-susceptible, virus-susceptible. The response of these strains to the two different kinds of infection, under the conditions of this laboratory, is epitomized by data presented in Table I. This table was compiled from data obtained during 1942 when the mice were in their 15th to 20th generation of brother-sister inbreeding. This system of inbreeding has been rigorously followed to the present. Although St. Louis encephalitis virus was not used in any of the experiments reported here, the mouse strain designations employed previously have been retained in this paper for the sake of uniformity with previous publications.

TABLE I

*Survivorship of Inbred Strains of Mice Selected for Resistance or Susceptibility to Infection with S. enteritidis or St. Louis Encephalitis Virus**

Description of strain	Strain designation	Test pathogen	No. of mice tested (in 1942)	Survivorship‡
				per cent
Bacteria-resistant, virus-resistant	BRVR	*S. enteritidis*	449	92
		St. Louis encephalitis	606	76
Bacteria-susceptible, virus-resistant	BSVR	*S. enteritidis*	86	2
		St. Louis encephalitis	98	88
Bacteria-resistant, virus-susceptible	BRVS	*S. enteritidis*	287	82
		St. Louis encephalitis	519	2
Bacteria-susceptible, virus-susceptible	BSVS	*S. enteritidis*	285	2
		St. Louis encephalitis	271	3

* Adapted from Schneider and Webster (1).

‡ Testing dose: *S. enteritidis*, 5,000,000 by stomach catheter; St. Louis encephalitis, 10^{-2} suspension of infected mouse brain, intranasally.

Pathogen.—Two cultures of *Salmonella typhimurium* (IV, V, VII: i-1, 2, 3) were used. One culture, TMO-S3, was relatively avirulent for uniformly susceptible mice. The second culture, BA_2SC-1, was highly virulent for mice. These cultures were derived (see reference 3) from cultures originally received from Dr. G. M. Mackenzie of the Mary Imogene Bassett Hospital of Cooperstown, New York. The present avirulent TMO and virulent BA_2 cultures of Mackenzie, although differing widely in virulence, were both smooth and indistinguishable in cultural, serological, immunizing, and toxigenic characters, and did not differ significantly in invasiveness and resistance to phagocytosis (Pike and Mackenzie (6)). All cultures were

perpetuated by preparing nutrient agar stabs with broth cultures of the organisms, incubating for 24 hours at 37.5°C., and then storing in the ice chest at 4°C. Fresh transfers were made at approximately monthly intervals.

Cultures were prepared for use in the infection experiments by seeding meat infusion broth tubes (10 ml.), incubating for 6 hours at 37.5°C., transferring a loopful to fresh broth (10 ml.) and incubating for 18 hours. The optical density of the cultures was then measured in an Evelyn photoelectric densitometer and the density of viable cells estimated (± 10 per cent) with the aid of a chart prepared from calibration data assembled for each of the two stock cultures. The appropriate dilutions were made with sterile saline, so that the desired dose of cells was suspended in a volume of 0.25 ml., which was injected intraperitoneally into each mouse. As a further check on dosage dilution plates were poured. In all instances these indicated that the administered dosage was as calculated from the densitometer data.

Environment.—All the experiments have been performed in the two air-conditioned rooms previously described (1). These rooms provide a constant temperature of 80 ± 0.5°F. and a constant relative humidity of 50 ± 3 per cent. The artificial lighting (fluorescent) is 12 hours per day, 6 a.m. to 6 p.m. The details of animal caging were the same as previously described (1).

Diet.—All the animals in these experiments were fed our standard laboratory stock diet plus distilled water *ad libitum*. This diet is Irwin's modification (4) of the Steenbock stock diet. It consists of the following items, listed in parts by weight: yellow corn meal, 64; linseed oil meal, 16; crude casein, 5; ground alfalfa meal, 2; powdered dried whole milk, 5; wheat germ, 10; dried yeast, 2; sodium chloride, 0.5; calcium carbonate, 0.5; cod liver oil, 2.

Under the environmental conditions already described the mortality risk of uninfected mice on this diet was nil from weaning age until the time when the experiments here to be described had been completed.

The Double Strain Inoculation Test Applied to BRVS and BSVS Mice

The opportunity to test the double strain inoculation method as a detector of differences in natural resistance due to genetic composition of the host was made possible by the presence in our laboratory of four different inbred strains of mice, the descendants of Webster's selected stocks (4, 5), which differed widely in their response to *Salmonella* infection, as measured by survivorship frequency. As Table I shows, two strains were "resistant" and two "susceptible." These divergent responses to infection were arranged by the geneticist's operation of inbreeding and selection, the guide to selection, be it noted, having been the response to an infection test performed with a single culture of *Salmonella*. The differences between these strains have been established as being innately, or genetically, determined (5). Indeed, the very fact that such stocks exist has been regarded by Zinsser, Enders, and Fothergill (7) as "the most trustworthy evidence which we possess at present for regarding it (inherited resistance) as a real phenomenon."

Before applying the double strain inoculation method to these inbred strains of mice it might be well to recapitulate the basic facts of the double strain phenomenon which underlie the experimental details. These relationships are well brought out by an examination of the effect of various time intervals between the initial admission of the avirulent pathogen population into the

mouse and the final admission of the virulent population. In such circumstances it has been found (3) that the frequency of survivorship increases rapidly with increase of the time interval between the avirulent and virulent doses. It was found further (3) that this rising curve of survivorship frequency was determined by the dietary status of suitable genetically heterogeneous mice, such curves rising more steeply if the mice were on a natural diet than when the mice were on a synthetic diet. The important point is that the DSI test in this manner revealed a divergence in the disease response of the mice on the two different diets. It followed, of course, that the mice which manifested the more steeply rising curve of survivorship frequency were designated as

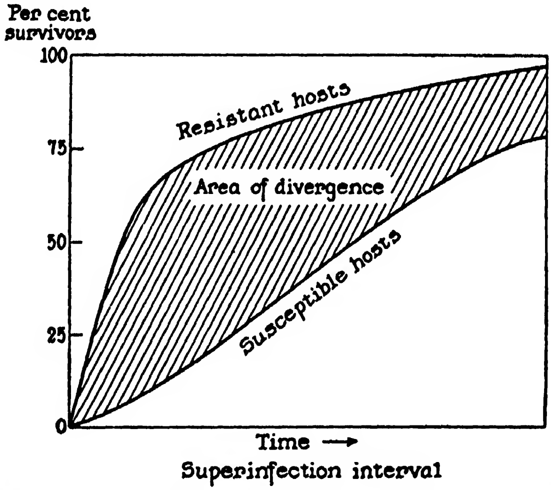


FIG. 1. Schematic diagram of the divergence, with time, of survivorship frequency of resistant and susceptible hosts as revealed by the double strain inoculation test.

"more resistant." The area described between these two rising curves of survivorship frequency is thus an effective index of the difference between the effects of the two diets on the mice at test. These considerations can be represented diagrammatically as in Fig. 1. Although in previous experiments (3) the area of divergence was due to the effect of diet difference (same mice on two different diets), this same divergence should emerge when the double strain inoculation test is performed with mice differing innately in natural resistance (two different kinds of mice on the same diet).

140 weanling BRVS mice (70♂, 70♀), and 140 BSVS mice (70♂, 70♀), were assembled in Room 1, as they became available, over a period of a month. During this period of assembly and throughout the course of the experiment the mice were fed the modified Steenbock stock diet and distilled water *ad libitum*. Four weeks after the last mice had been admitted to their respective assembly, the mice were transferred to Room 2 and placed in individual

cages. The mice were 2 to 3 months old at this point and the average body weight was as follows: BRVS, males 25.9 gm., females 22.1 gm.; BSVS, males 25.2 gm., females 21.4 gm.

Upon transfer to Room 2 the mice were divided by sex, age, and strain into 7 groups of 40 each, composed of subgroups of 20 BRVS and 20 BSVS mice. After 5 days of acclimatization to the new caging, the mice were infected with *S. typhimurium* in the following manner.

One group, the avirulent control, was injected intraperitoneally with 0.25 ml. of a sterile saline suspension of 10^3 viable cells of TMO-S3. A second group, the virulent control, was similarly injected with 10^5 BA_2SC-1. The third group was injected with 0.25 ml. of sterile

TABLE II

Survivorship of BRVS and BSVS Mice after Intraperitoneal Injection of Avirulent TMO-S3, Followed after Various Time Intervals by Virulent BA_2SC-1

Superinfection interval	Dose TMO-S3	Dose BA_2SC-1	Mouse strain	S/I*	%S‡	Survivorship difference	P
days						*per cent*	
Controls	10^3		BRVS	18/20	90	0	
			BSVS	18/20	90		
		10^5	BRVS	0/20	0	0	
			BSVS	0/20	0		
0	10^3	10^5	BRVS	0/20	0	0	
			BSVS	0/20	0		
1	10^3	10^5	BRVS	9/20	45	25	>0.1
			BSVS	4/20	20		
2	10^3	10^5	BRVS	15/20	75	50	<0.01
			BSVS	5/20	25		
7	10^3	10^5	BRVS	15/20	75	5	>0.9
			BSVS	14/20	70		
14	10^3	10^5	BRVS	19/20	95	25	>0.05
			BSVS	14/20	70		

* S/I, survivors/infected in test.

‡ %S, per cent survivors.

saline containing 10^3 TMO-S3 and 10^5 BA_2SC-1. On the same day groups 4 through 7 were injected with 10^3 TMO-S3 and after intervals of 1, 2, 7, and 14 days respectively, groups 4, 5, 6, and 7 were separately superinfected with injections of 10^5 BA_2SC-1.

All mice were observed for 30 days following the last injection of virulent BA_2SC-1 and were then discarded. Deaths were recorded daily. Mice which were alive at the end of the 30 day period were counted as survivors. Results are presented in Table II and Fig. 2.

The determining influence of the genetic constitution of the host for survivorship after double infection with virulent and avirulent *S. typhimurium* is clearly reflected in the data presented in Table II and, graphically, in Fig. 2. Just as in the diet experiments previously reported (3), survivorship is a func-

tion of the time experience of the avirulent *S. typhimurium*, in this instance for both strains of mice; but the resistant strain is characterized by the more rapid rate at which this increased frequency of survivorship is achieved. The designation then of the BRVS strain as a strain naturally resistant (relatively) to *Salmonella* infection is a valid one when tested by the DSI test; or conversely, if the BRVS strain is a satisfactory example of the phenomenon of natural resistance to infection, then the DSI test method is a valid method of identifying stocks of this sort. In order to confirm and extend this conclusion a second test was performed in which a second resistant strain of

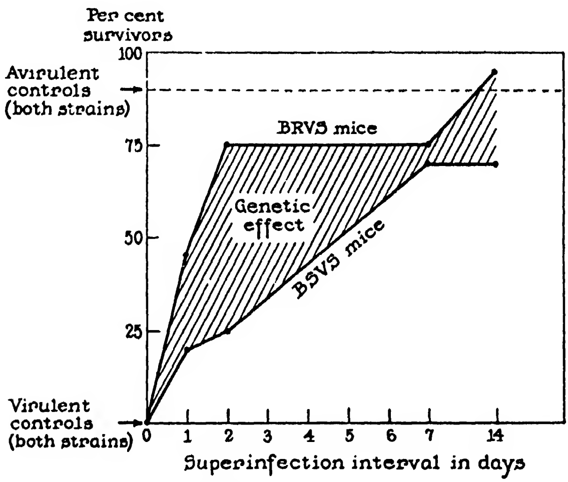


FIG. 2. Survivorship of BRVS and BSVS mice on a modified Steenbock diet after intraperitoneal injection of 10^3 avirulent TMO-S3 followed by 10^5 virulent BA_2SC-1 after various times.

mice, the BRVR strain, was compared with the same susceptible strain, BSVS, used above.

180 weanling BRVR mice (90 ♂, 90 ♀), and 180 weanling BSVS mice (90 ♂, 90 ♀) were assembled in boxes in Room 1, as they became available, over a period of 1½ months. During this period of assembly, and throughout the course of the experiment the mice were fed the modified Steenbock stock diet and distilled water *ad libitum*. Four weeks after the last mice had been admitted to their respective assembly, the mice were transferred to Room 2 and housed in boxes with beddings of wood shavings. At this transfer the mice were divided by sex, age, and strain into 9 groups of 40 each, composed of subgroups of 20 BRVR and 20 BSVS mice. They were housed in boxes of 10 each. After 5 days the mice were infected with *S. typhimurium* in the following manner.

One group, the avirulent control, was injected intraperitoneally with 0.25 ml. of a sterile saline suspension of 10^3 viable cells of TMO-S3. A second group, the virulent control, was similarly injected with 10^5 BA_2SC-1. The third group was injected with 0.25 ml. of sterile saline containing 10^3 TMO-S3 and 10^5 BA_2SC-1. On the same day groups 4 through 9 were

injected with 10^3 TMO-S3 and after intervals of 1, 2, 3, 7, 14, and 21 days respectively, groups 4, 5, 6, 7, 8, and 9 were separately superinfected with injections of 10^5 BA_2SC-1.

TABLE III

Survivorship of BRVR and BSVS Mice after Intraperitoneal Injection of Avirulent TMO-S3, Followed after Various Time Intervals by Virulent BA_2SC-1

Superinfection interval	Dose TMO-S3	Dose BA_2SC-1	Mouse strain	S/I*	%S‡	Survivorship difference	P
days						*per cent*	
Controls	10^3		BRVR	14/20	70	0	
			BSVS	14/20	70		
		10^5	BRVR	0/20	0	0	
			BSVS	0/20	0		
0	10^3	10^5	BRVR	0/20	0	0	
			BSVS	0/20	0		
1	10^3	10^5	BRVR	6/20	30	25	<0.1
			BSVS	1/20	5		>0.05
2	10^3	10^5	BRVR	15/20	75	50	<0.01
			BSVS	5/20	25		
3	10^3	10^5	BRVR	15/20	75	35	<0.1
			BSVS	8/20	40		>0.05
7	10^3	10^5	BRVR	15/20	75	−5	>0.7
			BSVS	16/20	80		
14	10^3	10^5	BRVR	12/20	60	−10	>0.5
			BSVS	14/20	70		
21	10^3	10^5	BRVR	16/20	80	−10	>0.5
			BSVS	18/20	90		

* S/I, survivors/infected in test.
‡ %S, per cent survivors.

All mice were observed for 30 days following the last injection of virulent BA_2SC-1 and were then discarded. Deaths were recorded daily. Mice which were alive at the end of the 30 day period were counted as survivors. Results are presented in Table III and Fig. 3.

It is apparent that the double strain inoculation procedure applied to a second inbred resistant strain of mice and compared with the same inbred susceptible strain has produced results which are similar to those reported above. As Fig. 3 shows, an area of divergence of survivorship frequency was

readily demonstrated and to that extent the claim that the DSI test is a test capable of revealing differences in natural resistance genetically determined is thereby strengthened.

It was next planned to study the relationship between a resistant strain and the second available susceptible strain, the BSVR strain. A difficulty arose, however, when it was found that the susceptibility of this BSVR strain is so great that when injected with 10^8 "avirulent" TMO-S3, all the mice died. Appreciable survivorship frequencies could be obtained only by reducing the dosage of TMO-S3 to 10^1, but this dose was so small, and the results were so erratic, it was feared that a large doubt had arisen whether, at such small

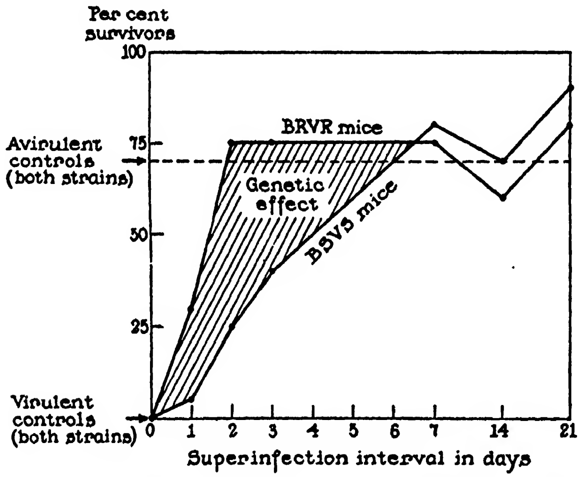


FIG. 3. Survivorship of BRVR and BSVS mice on a modified Steenbock diet after intraperitoneal injection of 10^8 avirulent TMO-S3 followed by 10^5 virulent BA_2SC-1 after various times.

dosages, all the animals were indeed experiencing a controlled contact with the bacterium. The DSI studies with BSVR mice were therefore abandoned. This experience, however, did bring to light a difference between the two susceptible strains, BSVR and BSVS, which had not been known to exist on the basis of the response to the original selection method with single cultures of *S. enteritidis*. (See Table I.)

DISCUSSION

From the data which have been presented above it is evident that those pre-existent host attributes present in mice which mitigate the effect of *Salmonella* infection, and which we designate as "natural resistance," are detectable by the double strain inoculation test whether those attributes are, in certain instances, determined by diet, or whether, in other instances, those attributes are genet-

ically determined. Although these facts may make permissible the use of the double strain inoculation test in studies of natural resistance, is this test a compulsory one? It would not appear to be so, for in Webster's hands (4, 5) a single culture of *Salmonella enteritidis* served satisfactorily for the selection of naturally resistant and susceptible mice. An explanation for this may be that Webster's cultures were not homogeneous, but heterogeneous and hence are not logically considered as *single* cultures, but as mixtures containing, in all probability, both virulent and avirulent cells. This is constructing an argument after the fact but it is a defensible argument as the following considerations will show.

In all the mouse infection tests which were necessarily performed during the approximately 10 year period that the resistant and susceptible stocks were being inbred and selected (4, 5) the bacterial tests were all performed with the same culture of *S. enteritidis* (*B. enteritidis*) bearing the laboratory designation of MT-1. This laboratory strain of MT-1 was first isolated by Lynch (8) in 1918 from a mouse epizootic in the breeding stock for cancer of the Rockefeller Institute. Twelve years later Webster began his breeding work and used this same culture which had been maintained by transfer at approximately monthly intervals and storage on nutrient agar in the ice chest. It is very unlikely, in the light of modern knowledge of bacterial dissociation, that a laboratory culture of this history could be considered as being homogeneous.

Further evidence that the laboratory culture of MT-1 was not homogeneous, but heterogeneous, was obtained in our laboratory in 1944 (2).

From these considerations it is apparent that a single dose of a mixed culture of pathogen variants can indeed serve as a method for measurement of natural resistance. Whether such a mixed culture serves satisfactorily will depend, among other things, on the relative proportions of the variants. In the zero time mixtures employed in the present experiments for example, the proportion of 10^3 avirulent and 10^5 virulent does not serve as a satisfactory single dose test since all the animals, irrespective of strain, died. This is probably not only a reflection of an imbalance between the two variants, weighted in favor of the virulent form, but also of other fundamental differences between the two variants which are concomitant with the artificial nature of their derivation and culture. The point is that by exploitation of the time curves a satisfactory relationship can be found. Historically, it would appear, there has been a dependence upon the usual heterogeneity of laboratory cultures for the testing of natural resistance differences. But this dependence places its reliance on factors left more or less uncontrolled, such as the relative proportions of the avirulent and virulent variants, to mention one factor. Such uncontrolled oscillations of variant frequencies, due to genetic drifts in the bacterial population, may account for Webster's experience with his inbred strains when he reported (5) "Control measures are frequently inadequate to prevent variation

in results such as the sudden increase of 15 to 20 per cent in mortality percentages in all lines recorded in the present experiments." Only the separate cultivation of avirulent and virulent variants makes possible the direct control of their respective frequencies in a mixture. However, when single cultures of unknown patterns of heterogeneity have been used, such instances can be regarded as constituting a special case of the double strain inoculation test; *i.e.*, the superinfection time interval has been reduced to 0. The utility of the double strain inoculation test is thus based, in part, on its generality and the DSI test is therefore not compulsory, but, for practical reasons, desirable.

SUMMARY

The double strain inoculation (DSI) method of testing for natural resistance to infection has been examined in the instance of mouse salmonellosis. The DSI method has been found capable of detecting differences in natural resistance due to genetic as well as nutritional causes.

A difference in response to *Salmonella* infection was found for the first time between the two "susceptible" inbred mouse strains, BSVR and BSVS. Whereas BSVS mice for the most part survived an intraperitoneal injection of 10^3 "avirulent" *S. typhimurium*, BSVR mice all succumbed.

The relationship of the DSI test to the usual single infection test has been discussed and it is suggested that such single infection tests are special cases of the DSI test, since they involve a heterogeneous bacterial population which can be considered as a mixture of cultures of differing virulence and in which, by a single injection, the usual time interval between the two injections of the DSI method has been reduced to 0.

BIBLIOGRAPHY

1. Schneider, H. A., and Webster, L. T., *J. Exp. Med.*, 1945, **81,** 359.
2. Schneider, H. A., *J. Exp. Med.*, 1946, **84,** 305.
3. Schneider, H. A., *J. Exp. Med.*, 1948, **87,** 103.
4. Webster, L. T., *J. Exp. Med.*, 1933, **57,** 793.
5. Webster, L. T., *J. Exp. Med.*, 1937, **65,** 261.
6. Pike, R. M., and Mackenzie, G. M., *J. Bact.*, 1940, **40,** 171.
7. Zinnser, H., Enders, J. F., and Fothergill, L. D., Immunity Principles and Application in Medicine and Public Health, New York, Macmillan Co., 1939, 110–111.
8. Lynch, C., *J. Exp. Med.*, 1922, **36,** 15.

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, June, 1949,
Vol. 179, No. 2, pp. 593–614]

ISOLATION OF SOME CRYSTALLINE YELLOW PEPTIDES FROM ENZYMIC DIGESTS OF DINITROPHENYL INSULIN AND DINITROPHENYL TRYPSINOGEN

BY D. W. WOOLLEY*

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, February 3, 1949)

Previous work has shown that the peptide-like growth factor strepogenin probably occurs in certain proteins such as insulin and trypsinogen at the amino end of the protein (1), and that it is liberated by suitable enzymic digestion. This conclusion suggested a means for attacking the almost insurmountable task involved in separating the one compound strepogenin from the mixture of numerous peptides and other cleavage products which result from the enzymic digestion of a protein. If the protein were converted to the dinitrophenyl (DNP) derivative by reaction of its amino groups with 2,4-dinitrofluorobenzene (2), the strepogenin of which the amino group is exposed should be converted into DNP strepogenin, which might then be liberated during enzymic digestion. A relatively small number of yellow, DNP compounds should result from such a digestion, because the number of amino groups of the proteins is small. Furthermore, the DNP derivatives are no longer amphoteric substances, but are organic acids which can be extracted into organic solvents and thus separated from the more numerous products of protein cleavage. This latter property very materially increases the scope of means available for successful separation of mixtures. For these reasons the isolation of pure, crystalline yellow cleavage products of DNP insulin and DNP trypsinogen has been attempted. Even though the biological inactivity of DNP strepogenin rendered impossible the direct determination of which, if any, of the isolated materials might be the derivative of the growth factor, the value of the study to the problem of the exact chemical structure of proteins seemed sufficient reason for proceeding.

Since much evidence points to the conclusion that strepogenin is a derivative of glutamic acid (3), the presence of this amino acid in one of the cleavage products of DNP insulin might indicate a relationship to strepogenin. However, all yellow peptides isolated contained this amino acid, and so no deduction could be made except that glutamic acid is very near the amino end of some of the peptide chains in this protein.

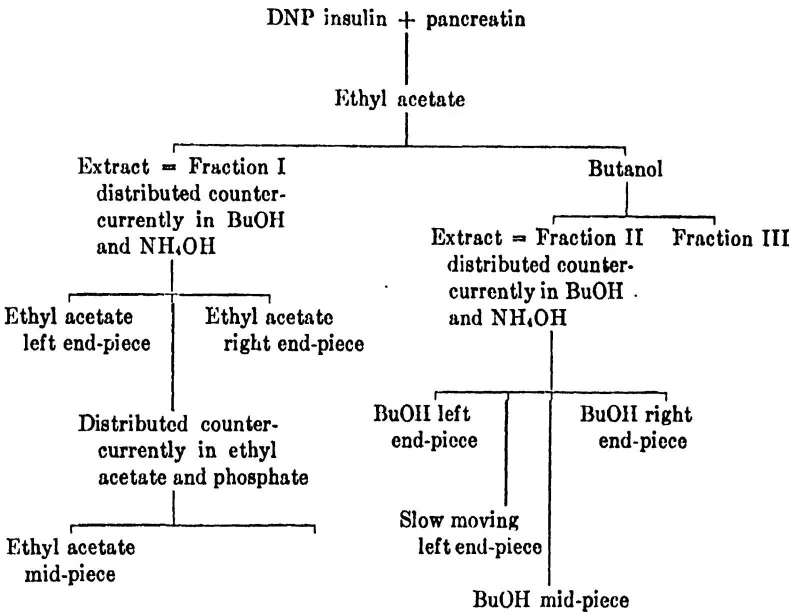
* With the technical assistance of R. A. Brown, J. Cowperthwaite, A. L. Holloway, and N. B. Smith.

When DNP insulin was digested with pancreatin and the digest acidified, three major fractions of colored material could be separated readily. One was obtained by extraction with ethyl acetate (Fraction I), a second by subsequent extraction of the residual aqueous phase with butanol (Fraction II), and a third remained suspended in the watery layer as an insoluble precipitate (Fraction III). Since Fraction III did not occur when the strepogenin-containing portion of oxidized insulin (1, 4) was used in place of the native protein, this fraction was not separated further, even though it amounted to more than half of the total DNP insulin. It was not merely undigested DNP insulin, because repeated treatment with fresh portions of enzyme did not destroy it, and yielded only small amounts of soluble components.

The ethyl acetate extract and the butanol extract were each separated into a number of components by a series of counter-current distributions between pairs of immiscible solvents. This method of separating pure compounds from mixtures of closely related substances has been developed by Craig *et al.* (5). For this purpose, no solvent system was found which was suitable for the separation of the free acids. Many which were tried, especially those containing chloroform, were found to cause extensive decomposition of the DNP peptides.[1] However, the partition of the ammonium salts between butanol and dilute aqueous ammonia proved quite useful and, in the case of several of the components, led to the isolation of crystalline, and apparently homogeneous compounds. Both the ethyl acetate extract and the butanol extract when separated in this fashion showed a colored fraction which did not move with the advancing front of butanol, and this was called the ethyl acetate left end-piece, or the butanol left end-piece, since it remained in the left-hand tubes of a series used in the counter-current distribution. A second set of colored tubes, found in the exact center of a counter-current series, was obtained from the butanol extract (or Fraction II). This was called the butanol mid-

[1] This rather unexpected behavior can best be illustrated by the following experiment. Fraction II (the butanol-extractable fraction) was distributed counter-currently between aqueous 5 per cent acetic acid and an organic layer made from 90 per cent chloroform and 10 per cent butanol. Part of the color remained completely in the aqueous phase and this fraction was partitioned counter-currently in a new solvent system of 5 per cent aqueous acetic acid and 1:1 chloroform-butanol. Most of the color remained completely in the organic layer. When this fraction was again distributed in the first solvent system, it was found to be so altered that all the color remained in the chloroform phase of the first tube, whereas before solution in the 1:1 chloroform-butanol it had remained in the aqueous phase. Indeed the color was now extractable into pure chloroform. This experience, together with many others of a similar character, indicated that exposure to acid, chloroform, and butanol appreciably altered the yellow compounds.

piece. In the ethyl acetate extract (Fraction I), a mid-piece, slightly to the right of the middle tube in a series, was found, and it was called the ethyl acetate mid-piece. At the right-hand end of a series of counter-current tubes of a partition of either the ethyl acetate extract (Fraction I) or of the butanol extract (Fraction II), there were some yellow fractions, representing substances which could not be extracted from butanol solution into aqueous ammonia. These were designated the ethyl acetate and butanol right end-pieces. In addition, there was a colored substance which was present in the butanol extract or Fraction II which moved only



slowly down a series of counter-current tubes containing butanol and dilute aqueous ammonia, and this was called the slow moving butanol left end-piece. A flow sheet to clarify the preparation of these major fractions is shown.

Some of these fractions obtained by counter-current distribution appeared either to be pure compounds or ones which could be rendered pure by simple additional distributions. This was true for the butanol mid-piece, the ethyl acetate mid-piece, and the slow moving butanol left end-piece. The criteria of purity were the following: (*a*) Each compound moved as a single symmetrical peak in counter-current partition of the ammonium salts between butanol and dilute aqueous ammonia. For each substance the symmetry of the peak was demonstrated by examination of

tubes on the left-hand side and on the right-hand side of the one with maximal color. When the contents of these tubes were distributed counter-currently in the same solvent system, they showed a maximal color in the identical position to that assumed by the peak in the previous counter-current series. If readily separable impurities had been present, the position of the peak in the secondary counter-current distributions would not have been the same as in the primary one. (*b*) Evaporation of the ammoniacal solution of each of the isolated fractions left crystalline yellow solids with rather characteristic and reproducible melting points. (*c*) Counter-current distribution of the isolated fractions in new solvent systems failed to indicate the separation of a colored impurity. Although two compounds might have the same rate of migration in one pair of solvents, the likelihood that they would also move at the same speed in a second pair of solvents is more remote. (*d*) Because one might argue that the number of transfers in the distributions was not large enough to insure adequate separation, each isolated fraction was placed on a paper strip chromatogram (6) and examined in two different solvent systems. One of these was phenol saturated with dilute aqueous ammonia and the other was butanol saturated with dilute aqueous ammonia. There is evidence from many prior investigations that the resolving power of paper strip chromatograms is quite good. In the solvent systems employed the isolated fractions behaved as single substances. (*e*) Each isolated fraction was hydrolyzed and the hydrolysates were analyzed quantitatively for various amino acids by microbiological procedures. These tests showed that the molecular ratios between the amino acids in each substance were approximately 1:1. In the slow moving butanol left end-piece approximately 3 moles of leucine were present for each mole of the other amino acids. If the isolated fractions were mixtures, one would not expect such stoichiometric proportions unless the components of the mixture were present in equimolecular amounts. The probable error in microbiological assays is large, but despite this, gross impurities should have been detected by the procedure outlined above.

These criteria of purity may not be sufficient for substances such as peptides, but they are possibly as good as any thus far applied to such materials. Taken together they indicate that the isolated substances were of sufficient homogeneity to warrant further study.

A few of the fractions obtained by counter-current distribution were impure. This was true of the ethyl acetate right end-piece which formed a diffuse band of yellow color spread over several of the tubes at the right-hand end of the counter-current series. In these cases, some separation was achieved by differential solvent extraction and by extended counter-current distribution in new solvent systems, but the obtaining of homogeneous material from the end-pieces was not achieved.

The right-hand end-pieces, especially the butanol right end-piece, contained some artifacts. These fractions, it will be recalled, represented those substances which were completely held in butanol in preference to aqueous ammonia. They must, therefore, have been ammonium salts with a far greater affinity for butanol than for water, or else they were neutral compounds with no carboxyl groups. Now, since the peptides in the butanol right end-piece contained serine and threonine, the hydroxyls of these amino acid residues in the peptide might be so situated as to allow lactone formation with the carboxyl groups. In this way neutral compounds could result. Evidence for this hypothesis was found in that the dry substances in the butanol right end-piece would not dissolve in 0.1 N NaOH. However, when heated in this solvent for a time, solution occurred. When the resulting solution was acidified, the free acid was formed, and this, when distributed counter-currently between butanol and dilute aqueous ammonia, showed clearly two colored compounds. One of these moved in counter-current series as did the butanol mid-piece, and the other as did the butanol right end-piece. The amino acid composition of the fractions was identical, as determined by the paper strip method of Consden, Gordon, and Martin (6), and was not altered from that of the original butanol right end-piece. During the acidification following the opening of the lactone ring by gently heating with alkali, some of the free acid formed reverted to the lactone, which once again appeared as a neutral compound at the right-hand end of a counter-current series. Furthermore, if the butanol mid-piece, isolated in the normal course of fractionation of the DNP insulin digest, was dissolved in water, acidified, and extracted with butanol, a counter-current partition of the extract so obtained showed not only a mid-piece as expected, but also a right end-piece, which probably arose by lactonization while the substance was in acid solution. The qualitative amino acid composition was unchanged during these operations. In view of these facts it would seem probable that at least some of the butanol right end-piece was an artifact which arose from the butanol mid-piece by lactonization while the latter was exposed to acid. This instability of the free acids, as well as other less clearly defined decompositions and rearrangements of the fractions under investigation, made it imperative to employ the mildest conditions possible for their preparation.

The amino acids obtained by hydrolysis of each pure DNP peptide were determined qualitatively by paper strip chromatography, and the existence of most of the amino acids so indicated in the hydrolysates was confirmed microbiologically (7). The ethyl acetate left end-piece, after suitable purification, contained a substance which, when hydrolyzed, yielded only glutamic acid and the chromophoric group. The ethyl acetate mid-piece gave rise to the chromophoric group, glutamic acid, serine, threonine,

alanine, valine, leucine, and isoleucine. The butanol mid-piece yielded these same constituents and, in addition, aspartic acid. The slow moving butanol left end-piece gave the same amino acids (except alanine) as the butanol mid-piece, and the butanol left end-piece showed all except threonine, of the constituents of the butanol mid-piece plus cystine. The existence of serine and alanine in these substances was demonstrated solely by paper strip chromatography. While the serine could be detected with considerable certainty, alanine was not always easy to differentiate from the threonine, and for this reason there was some doubt of its existence in the compounds.[2] The demonstration of the coexistence of leucine and isoleucine depended entirely on microbiological assay, since these two amino acids were not separable by chromatography on paper strips. Because the detection of the amino acids depended on the paper strip method, some of them may have escaped notice and thus the entire qualitative composition of the yellow peptides was not regarded as established with certainty.

The chromophoric group in each of the fractions was tentatively concluded to be a DNP-glycine residue, but the evidence for this was unsatisfactory. It consisted of the isolation of DNP-glycine from acid hydrolysates, but the difficulty was that the yield was very low, and most of the colored hydrolysis product was obtained as an unidentified mixture which could be separated in a series of counter-current distributions between butanol and dilute aqueous ammonia as a left end-piece, which did not migrate with the butanol, and a right end-piece, which did not remain at all in the aqueous phase. This latter component was a neutral material.

The failure to isolate good yields of DNP-glycine was not due to inadequacies of the methods, because the counter-current distribution procedure was found to be an elegant way to separate DNP-glycine and DNP-phenylalanine, both of which Sanger has shown to occur in acid hydrolysates of DNP insulin (2). However, in contrast to Sanger's findings with a chromatographic method of separating DNP amino acids from hydrolysates of DNP insulin, the counter-current procedure when applied to such hydrolysates showed a third colored substance, corresponding in position to the right end-piece yielded by hydrolysates of the peptides isolated in this work. When pure DNP-glycine was subjected to the action of hot 20 per cent HCl, such as is used in the hydrolysis of the peptides, a minor portion of it was converted into a colored substance which stayed at the right-hand end of a counter-current series, but the extent of this decomposition was small compared to that found with the DNP peptides isolated in

[2] For this reason the composition of two of these peptides which were described in a preliminary note (11) was slightly in error. The use of ammonia in the paper strip apparatus permitted a better means of distinguishing between threonine and alanine than did neutral aqueous phases.

this work. Studies with some synthetic peptides containing DNP-glycine residues revealed one which yielded no DNP-glycine when hydrolyzed with hot 20 per cent HCl, but which gave instead two colored components as did the compounds from insulin. Thus, bis-DNP-diglycyl-α-aminoalanine, although containing a high amount of DNP-glycine residues, gave no DNP-glycine when boiled with strong HCl. This synthetic substance was converted completely into degradation products similar to those from the isolated peptides. This fact tended to show that DNP-glycine can exist in a peptide in such a linkage as to preclude its liberation by the ordinary acid hydrolysis; but it does not imply that a structure identical with the synthetic model compound existed in the peptides from insulin. The small yield of DNP-glycine which was isolated from hydrolysates of the insulin fractions may be taken as evidence that the chromophore in these substances was DNP-glycine; or it may be taken as indication that the peptides were contaminated with small amounts of impurities which gave rise to to DNP-glycine.

Insulin may be cleaved, as Sanger has shown (4), by oxidation with performic acid in formic acid solution, and when this is done, two major fractions are obtained. One fragment is soluble in water at pH 6, and the other is not. The cleavage appears to depend on rupture of the molecule by oxidation of —S—S— bridges of cystine residues rather than to hydrolysis of peptide bonds. Sanger has reported that the fraction soluble at pH 6 owes its free amino groups to glycine, and that the basic amino acids seem to be absent from it. Since Woolley (1) has shown that the strepogenin activity of insulin resided in this fraction of the oxidized protein, the DNP derivative of it was prepared, digested with pancreatin, and the digest was fractionated by the methods described for DNP insulin. Two major differences were noted in the yellow peptides from oxidized insulin fraction and those from DNP insulin: (1) practically no Fraction III (the yellow residue not extractable by ethyl acetate or by butanol) appeared, and (2) the butanol left end-piece gave a compound containing cysteic acid instead of cystine.

Pancreatin digests of DNP trypsinogen were fractionated by the same methods as were used for DNP insulin, and two yellow peptides indistinguishable from those from DNP insulin were isolated. These were the butanol mid-piece and the ethyl acetate mid-piece. The probable identity of the fragments from insulin with those from trypsinogen was indicated by the following evidence: (1) The melting points were the same. (2) The positions in series of counter-current distributions were the same. This was true not only in butanol and dilute aqueous ammonia but also in other solvent systems. (3) The compounds from DNP insulin moved at the same rate as those from DNP trypsinogen on paper strip chromatograms when tested side by side on the same paper. (4) The qualitative

amino acid composition as revealed by paper strip chromatography of hydrolysates was identical for the corresponding fractions of the two proteins. Thus it would seem that two moderately large pieces of both proteins were identical. Whether the same fragments could be found in other proteins not originating in the pancreas is a question of importance to investigate.

A third yellow peptide was isolated from digests of DNP trypsinogen. This one yielded DNP-methionine, serine, and isoleucine when it was hydrolyzed with acid. No evidence was found of a similar substance in digests of DNP insulin.

Because the yellow peptides isolated from DNP insulin seemed to form a regular series of increasing amino acid complexity, the hypothesis might arise that they were all derived by graded hydrolysis from a single portion of the protein molecule. The order of occurrence of the amino acid residues, counting from the free amino group of the protein, might thus be deduced, at least in part. This hypothesis has been examined in the following ways and found not to fit the observed facts. The more complex peptides were digested further with fresh quantities of pancreatin, and the digests were examined for the presence of the less complex substances which one would expect to arise by stepwise degradation of the larger molecules. The slow moving butanol left end-piece was found not to give smaller yellow fragments when digested further. The butanol mid-piece, on the other hand, did yield small amounts of what appeared to be the ethyl acetate left end-piece and the ethyl acetate mid-piece; therefore, the existence of two differing large peptides was indicated among the compounds isolated.

A second line of evidence pointed to the same conclusion. Partial hydrolysis with cold, concentrated HCl gave a yellow cleavage product from the slow moving left end-piece and analysis showed that this fragment contained the chromophore, leucine, isoleucine, and glutamic acid. Similar treatment of the other large peptides (*e.g.* the butanol mid-piece) gave a new fragment containing only the chromophore and glutamic acid. Thus, again, the existence of two large peptides was indicated with differing arrangements of amino acid residues.

It would seem that at least two distinct peptide chains in insulin gave rise to the products isolated in this study. Much concerning the order of amino acids in this protein may be learned by further investigation of these materials.

EXPERIMENTAL

Pancreatin Digestion of DNP Insulin and Preliminary Separation of DNP Cleavage Products with Solvents—A typical run will be described in

this and subsequent sections to illustrate the procedures employed. 1.1 gm. of DNP insulin prepared according to Sanger's directions (2) from recrystallized insulin[3] were suspended in 600 cc. of water containing 2.5 gm. of K_2HPO_4, and the suspension was heated. When it was almost boiling, the protein suddenly dissolved.[4] The cooled solution was treated with 100 mg. of pancreatin, covered with a thin layer of toluene, and held at 37° overnight. Enough HCl was added to reduce the pH to 3, and the resulting suspension was extracted five times[5] with 500 cc. portions of ethyl acetate. The extracts were evaporated under reduced pressure to dryness at a temperature below 40°. This was Fraction I. The aqueous suspension remaining after the ethyl acetate extraction was extracted four times with 400 cc. portions of butanol, and the extracts were mixed with 1 liter of water and 10 cc. of concentrated ammonium hydroxide and concentrated under reduced pressure to dryness. Care was taken that the water was the last solvent to evaporate, because anhydrous butanol was deleterious. This was Fraction II. The residual aqueous suspension after the butanol extraction was filtered, and the precipitate was washed well with water and dried to yield 700 mg. This was Fraction III. The aqueous filtrate was colorless.

Isolations of Pure DNP Peptides by Counter-Current Distribution of Fractions I and II—For these separations, the general method of Craig *et al.* (5) was followed. The labor-saving machine developed by Craig was not used because it was necessary to centrifuge each tube after each transfer in order to cause the solvents to clear. The distributions were performed in centrifuge tubes, and the top layer was moved from tube to tube down the series with a fine tipped pipette.

Fraction I (the ethyl acetate extract) was dissolved in 15 cc. of water containing 5 per cent of its volume of concentrated ammonium hydroxide, and the solution was evaporated under reduced pressure to dryness and counter-currently distributed between butanol and dilute aqueous ammonia (95 cc. of water plus 5 cc. of concentrated ammonium hydroxide) through a total of twenty tubes. 15 cc. of each solvent were used in each tube. The color in each tube was judged visually, and maxima, or peaks, were found in Tubes 1 (left end-piece), 12, 13, and 14, and a broad one in Tubes 16, 17, 18, 19, and 20.

[3] Crystalline insulin used in this work was very kindly supplied by Eli Lilly and Company, by Hoffmann-La Roche, Inc., and by E. R. Squibb and Sons. Most of the experiments were performed with a Lilly sample, derived from beef, which had been recrystallized several times.

[4] Because the DNP insulin would only dissolve in rather hot alkaline solution, some change in the protein caused by these conditions seems probable.

[5] The extraction was continued until the final extract was colorless. The ethyl acetate mid-piece was not readily extractable into ethyl acetate.

Ethyl Acetate Left End-Piece—The contents of Tube 1 were concentrated under reduced pressure to dryness, dissolved in 15 cc. of water, and the solution was adjusted to pH 3 with HCl and extracted twice with ethyl acetate. The extracted material was dried under reduced pressure, then triturated with 10 cc. of ethyl acetate, and the insoluble matter was discarded. The soluble portion when evaporated left 6 mg. of a rather hygroscopic, crystalline residue. This was the ethyl acetate left end-piece.

Ethyl Acetate Mid-Piece—To purify the ethyl acetate mid-piece, the contents of Tubes 10 to 14, containing the middle peak in the counter-current series, were combined, concentrated under reduced pressure to dryness, and the residue was counter-currently distributed between ethyl acetate and a buffer made from 1 volume of 0.1 M Na_2HPO_4 and 2 volumes of 0.1 M NaH_2PO_4. 15 cc. of each solvent were used in each tube and the fraction was distributed through ten tubes. A small amount of impurity, identified as 2,4-dinitrophenol,[6] m. p. 116°, was thus removed as a peak in Tubes 4, 5, and 6, and the pure ethyl acetate mid-piece was recovered from Tubes 1, 2, and 3 (peak in Tube 2) by acidification to pH 3, extraction with ethyl acetate, and counter-current distribution between butanol and dilute aqueous ammonia. This last was done in order to obtain the ammonium salt, because of the hygroscopic and unstable nature of the free acid. By evaporation of the contents of the tubes showing color in this distribution, a crystalline preparation weighing 10 mg. was secured. These crystals lost birefringence and appeared to melt at 163°.[7] After this change a solid residue was left which did not liquefy below 230°.

In an attempt to purify the substance further, it was distributed counter-currently between butanol and 5 per cent aqueous ammonium hydroxide through twelve tubes with 30 cc. of the aqueous phase and 10 cc. of the butanol in each tube. A symmetrical peak of color appeared in Tube 7. In order to test the symmetry of this peak the contents of Tube 5 were distributed counter-currently in the same solvent system. A single peak of color occupying the same position in the series as that in the primary distribution was observed. Similarly, when the contents of Tube 9 of the primary distribution were distributed counter-currently, a single peak of color in the same position as that of the primary series was found.

0.02 cc. of a 0.1 per cent solution of the colored substance in 3 per cent aqueous ammonium hydroxide was placed on a strip of Whatman No. 1

[6] If the acidified pancreatin digest was extracted with ether before ethyl acetate was used, the dinitrophenol was effectively removed. When this modification was used, the definition of the position of the mid-piece in the distribution was much clearer and the use of the phosphate-ethyl acetate counter-current distribution step was unnecessary.

[7] All melting points reported in this paper were determined on a hot stage microscope.

filter paper and the strip was treated as a paper chromatogram according to the general procedure described by Consden *et al.* (6). When the solvent in the boat was phenol saturated with 3 per cent aqueous ammonium hydroxide, the yellow color of the compound was observed to move as a single spot with an R_F of 0.68. When the solvent was butanol saturated with 3 per cent aqueous ammonium hydroxide, the color moved as a single spot with an R_F of 0.63.

Separation of Fraction II; Butanol Left End-Piece—Fraction II, the butanol extract of the digest, was separated by counter-current distribution between butanol and dilute aqueous ammonia through a series of fourteen tubes with 15 cc. of each phase in each tube. Maximal color was in Tube 2, in Tube 7, and in Tubes 13 and 14. In order to separate further the components at the left-hand end, the contents of Tubes 1 to 4 were freed of solvents under reduced pressure and distributed counter-currently between butanol and dilute ammonia through twelve tubes, with 30 cc. of butanol and 10 cc. of aqueous phase in each tube. In this way the slow moving butanol left end-piece was concentrated in Tubes 3 and 4 and was separated from the butanol left end-piece which remained in Tube 1. This latter was precipitated as the free acid by evaporation of the solvents, solution in water, and addition of HCl. The only evidence for its purity was that preparations made from successive batches of digest showed the same decomposition point of 220°, and that on a paper strip chromatogram with butanol-ammonia the color did not move, while with phenol-ammonia a single spot of R_F 0.58 appeared.

Slow Moving Butanol Left End-Piece—The slow moving butanol left end-piece was obtained as 30 mg. of rod-shaped crystals, melting at 220° after darkening from 196°, by evaporation of the solvents under reduced pressure. Attempts were made to purify it further by counter-current distribution between butanol and phosphate buffer, but no heterogeneity was found. Similarly, it was purified by counter-current distribution between butanol and 5 per cent aqueous solution of butylamine. In this system, the butylamine salt of the peptide migrated faster than the ammonium salt had done in the runs above. With equal volumes of the two phases, a homogeneous peak was found in Tube 3 of a series of ten tubes. The butylamine salt was oily when dried. The homogeneity of the colored material in the counter-current distribution series with butanol and aqueous ammonium hydroxide was established in the same way as was described for the ethyl acetate mid-piece. When the compound was tested on paper chromatograms, it showed a single spot of yellow color with an R_F of 0.12 in butanol-ammonia and a single spot of R_F 1.0 in phenol-ammonia.

Butanol Mid-Piece—The butanol mid-piece was freed of a trace of impurity by counter-current distribution between butanol and 0.1 M

sodium phosphate buffer made from 4 volumes of Na_2HPO_4 and 1 volume of NaH_2PO_4 in a series of eight tubes. The impurity was found in Tubes 3 to 5, but it was so small in amount (2 mg.) that its presence did not materially affect the melting point of the main fraction. This latter was recovered from Tubes 7 and 8 by acidification to pH 4, extraction with butanol, and counter-current distribution between butanol and dilute ammonia. This latter step was necessary because in the acidification some of the substance was changed to that occurring in the butanol right end-piece (lactone of the mid-piece?). The mid-piece, obtained by evaporation of the ammoniacal solution, was a crystalline substance (26 mg.). When heated, it changed rather sharply at 135°, but when heating was continued, a second change occurred at 160° and the compound finally melted at 205–210°.

The symmetry of the peak of color occurring in the counter-current distribution series with butanol and dilute aqueous ammonia was demonstrated in a manner similar to that described for the ethyl acetate mid-piece. When the compound was tested on paper strip chromatograms, it was found to move as a single spot with an R_F of 0.50 in butanol-ammonia and with an R_F of 0.86 in phenol-ammonia. The presence of the impurity which was removed by the distribution in butanol and phosphate buffer could be demonstrated readily in crude preparations on a paper strip chromatogram with phenol-ammonia, for this impurity had an R_F of 0.68 in this solvent. On a butanol-ammonia chromatogram the impurity could not be distinguished from the butanol mid-piece.

Isolation of DNP Peptides from pH 6 Soluble Portion of Oxidized Insulin—Since details of the procedure used by Sanger (4) for the oxidation of insulin and the separation of the fragments are not yet available, the method used in this work will be described. 500 mg. of crystalline insulin[8] were dissolved in 30 cc. of commercial formic acid, and 3 cc. of a 30 per cent solution of hydrogen peroxide were added. After 15 minutes, the formic acid was removed as completely as possible in a good vacuum at 40°, the glassy residue was rubbed with 35 cc. of 0.2 N H_2SO_4, and the suspension was freed of H_2O_2 by alternate additions of small portions of KI and of $Na_2S_2O_3$. The mixture was adjusted to pH 6, and after 2 days in the cold it was filtered and the precipitate was washed with 2 cc. portions of water. The filtrate was concentrated under reduced pressure to about 30 cc. and 600 mg. of $NaHCO_3$ were added. Reaction with 2,4-dinitrofluorobenzene (0.75 cc.) was conducted according to the general procedure of Sanger. After extraction of the excess reagent with ethyl acetate at pH 6, the DNP derivative was precipitated with HCl, washed, and dried.

[8] Squibb insulin was used.

Pancreatin digestion of the product and separation of the yellow peptides were carried out as in the case of DNP insulin. Only 10 mg. of Fraction III (not extracted by ethyl acetate or by butanol) were found. In the counter-current distributions, the ethyl acetate mid-piece, the butanol mid-piece, and the slow moving butanol left end-piece were obtained from the peaks which occurred in the same positions in the series as were found in the case of DNP insulin. The melting points of these compounds were the same as those reported above. Impure colored fractions occurred at both left and right ends in the distribution of both Fractions I and II.

Isolation of Ethyl Acetate Mid-Piece and Butanol Mid-Piece from Digests of DNP Trypsinogen—Crystalline trypsinogen[9] was converted to the DNP protein by the same procedure as that used with insulin (2). DNP trypsinogen, in contrast to DNP insulin, would not dissolve in cold or hot solutions of K_2HPO_4, and in order to bring it into solution, heating to boiling in 0.1 N NaOH for a minute was required. The degradation of the protein by this drastic procedure may have been extensive. As soon as solution was effected, the pH was lowered to 8. 1 gm. of DNP trypsinogen was thus dissolved in 400 cc. of water and digested with 100 mg. of pancreatin at 37° overnight. The solution was buffered with 500 mg. of K_2HPO_4 during this operation. The digest was then acidified to pH 3 and treated in the same manner as that described for DNP insulin. Fraction I (the ethyl acetate extract) yielded an ethyl acetate mid-piece, the position of which in the counter-current distribution series was identical to that for the ethyl acetate mid-piece of insulin. This fraction behaved the same as that from DNP insulin in the counter-current distribution with ethyl acetate and phosphate buffer. The symmetry of the peak of color, found in a counter-current distribution of the purified fraction through fourteen tubes of butanol and dilute aqueous ammonia, was established in the same way as that described for the ethyl acetate mid-piece of DNP insulin. The purified compound from DNP trypsinogen showed the same R_F values as did the corresponding fraction from DNP insulin when tested on paper strips with butanol-ammonia and with phenol-ammonia. When the fraction from DNP trypsinogen was tested on the same paper strip with that from DNP insulin, the two substances were found to move at the same rate. The melting point of the compound isolated from DNP trypsinogen was the same as that described for the ethyl acetate mid-piece from DNP insulin. The yield of purified material was 10.6 mg.

Fraction II (the butanol extract of the digest) was treated in the manner described for the corresponding fraction from DNP insulin. A peak of color appeared in the same position in the counter-current distribution as that occupied by the butanol mid-piece from DNP insulin. This colored

[9] Crystalline trypsinogen very kindly supplied by Dr. M. Kunitz of this Institute.

fraction was purified by counter-current distribution between butanol and phosphate buffer and it was found to behave exactly as did the butanol mid-piece from DNP insulin. The purified substance from the butanol-phosphate distribution was distributed counter-currently between butanol and dilute aqueous ammonia through twenty tubes, and it appeared as a single colored peak with a maximum in Tube 10. The symmetry of this peak was demonstrated by secondary counter-current distributions, as have been described above. The purified substance was tested on paper strip chromatograms and was found to have the same R_F values in butanol-ammonia and in phenol-ammonia as those described for the butanol mid-piece from DNP insulin. These determinations were carried out simultaneously with those for the substance from insulin. The melting point of the compound from DNP trypsinogen was the same as that described for the butanol mid-piece from DNP insulin. The yield was 5.5 mg.

Isolation of New DNP Peptide from DNP Trypsinogen—When Fraction I (ethyl acetate extract) of the DNP trypsinogen digest was distributed counter-currently between butanol and dilute aqueous ammonia, a new colored substance appeared which had not been found in the digest of DNP insulin. This substance showed a peak of color with a maximum in Tube 3 of a series of fourteen. Tubes 2, 3, and 4 were combined, the solvent was removed under reduced pressure, and the residue was purified by counter-current distribution through twelve tubes, each containing 7.5 cc. of butanol and 2.5 cc. of 5 per cent aqueous ammonium hydroxide. A symmetrical peak with a maximum in Tube 6 was found. The symmetry of this peak was demonstrated by secondary counter-current distributions such as those described above. The colored substance moved as a single spot on paper strip chromatograms. The R_F of this spot was 0.20 with butanol-ammonia and 0.68 with phenol-ammonia. When the solutions were evaporated, the substance tended to remain oily and crystallized only after prolonged storage. The yield was 4.1 mg.

Amino Acid Composition of DNP Peptides—To determine the qualitative amino acid composition of a fraction, about 1 mg. was refluxed with 15 cc. of 20 per cent HCl for 24 hours, and the hydrolysate was evaporated to dryness under reduced pressure repeatedly, neutralized, and made to 1 cc. 0.02 cc. was analyzed on strips of Whatman No. 1 filter paper according to the directions of Consden, Gordon, and Martin (6). Phenol was the most satisfactory solvent tested, and it was used routinely. Occasionally butanol, or a mixture of butanol and benzyl alcohol, or collidine, was also used. All fractions were examined with phenol saturated with water and with phenol saturated with 3 per cent aqueous ammonium hydroxide. As recommended by the originators of the method, a known mixture of amino acids approximating the composition of the unknown was always run beside the unknown on the same strip of paper.

In the case of fractions judged to be homogeneous (such as the mid-pieces) the results of the paper strip analysis were confirmed by microbiological assays. The qualitative composition of these substances is shown in Table I. The quantitative results of microbiological assay are shown in Table II. The values in Table II serve only to indicate the molecular proportions of amino acids in the hydrolysates and do not represent percentages of these constituents in the peptides. In most cases the

TABLE I

Qualitative Amino Acid Composition of Purified DNP Peptides as Judged by Paper Strip Chromatography and by Microbiological Assay

Description of fraction	Chromophore	Glutamic acid	Serine	Threonine	Alanine	Leucine	Isoleucine	Valine	Aspartic acid	Cystine
From DNP insulin										
1. Ethyl acetate left end-piece .	+	+								
2. " " mid-piece. .	+	+	+	+	+	+	+	+		
3. Butanol mid-piece. . . .	+	+	+	+	+	+	+	+	+	
4. Slow moving butanol left end-piece.	+	+	+	+		+	+	+	+	
5. Butanol left end-piece.	+	+	+		+	+	+	+	+	+
6. HCl cleavage product of (4).	+	+				+	+			
7. " " " " (3)*†. . .	+	+								
From DNP trypsinogen										
8. Ethyl acetate mid-piece	+	+	+	+	+	+	+	+		
9. Butanol mid-piece	+	+	+	+	+	+	+	+	+	
10. Ethyl acetate slow piece*.	+		+				+			

* Traces of the other amino acids contained in Peptide 3 could be detected if a high concentration of hydrolysate was tested.

† Not the same as Peptide 1 because it moved at a faster rate in counter-current distribution (*cf.* the text).

amino acids were in equimolecular proportions. However, in the slow moving butanol left end-piece, the ratio of leucine to the other components was about 3:1. Valine, in this peptide, was too high for a 1:1 ratio. Aspartic acid, isoleucine, and threonine were determined with the aid of *Leuconostoc mesenteroides* and glutamic acid, leucine, and valine with *Lactobacillus arabinosus*. Threónine was also determined with *Streptococcus faecalis*. The same basic amino acid composition in the medium (8, 9) was used in all instances, and the particular acid to be determined was omitted for the assay. A micro modification of the usual procedures

was employed in which a total volume of 1 cc. of medium was used per tube.

Nature of Chromophoric Group. (a) Counter-Current Distribution of Synthetic DNP Compounds—2 mg. samples of various synthetic DNP compounds were distributed counter-currently between butanol and dilute aqueous ammonia through a series of eight tubes. 5 cc. of each solvent were used in each tube. The characteristic rate of migration of each of the compounds tested is shown in Table III. Values for other solvent systems are also given.

TABLE II

Amounts of Various Amino Acids Found in Hydrolysates of DNP Peptides

The results are in micrograms per cc. of hydrolysate and do not represent percentages in the peptides.

Peptides	Aspartic acid	Glutamic acid	Threonine	Valine	Leucine	Isoleucine
From DNP insulin						
1. Ethyl acetate mid-piece....	0	50	42	39	46	52
2. Butanol mid-piece... ...		150		124	140	120
3. Slow moving butanol left end-piece..	25	25	19	28	68	
4. Butanol left end-piece............ ..		85		78	72	80
5. HCl cleavage product from (3).. ..		70			60	60
From DNP trypsinogen						
6. Ethyl acetate mid-piece..............	0		24	35	37	
7. Butanol mid-piece..........			73	90	101	

(b) Isolation of DNP-Glycine and DNP-Phenylalanine from DNP Insulin—50 mg. of DNP insulin were refluxed in 15 cc. of 20 per cent HCl for 4 hours, and the acid was removed under reduced pressure. The residue was suspended in water, and the suspension was extracted four times with ethyl acetate. The extract was freed of solvent and partitioned counter-currently between butanol and dilute aqueous ammonia through eight tubes with 5 cc. of each solvent in each tube. Maxima of color, or peaks, appeared in Tubes 3, 6, and 8. The contents of Tubes 2 and 3 were freed of solvents and counter-currently distributed between ethyl acetate and 0.1 M acetate buffer made from 1 volume of acetic acid and 3 volumes of sodium acetate. When the distribution was continued through eight tubes, a good peak was found in Tube 4. The contents of Tubes 3 to 5 were acidified, and the ethyl acetate layers were evaporated. The residue was washed with water, dried, and shown to be DNP-glycine by its melting point of 205°. DNP-phenylalanine was obtained from Tubes 6 and 7 of

TABLE III

Position of Various Synthetic Compounds in Counter-Current Distribution between Immiscible Solvents in Series of Eight Tubes with Equal Volumes in Both Layers

Compound	Tube No., showing maximal color			
	With butanol and aqueous NH_4OH*	With ethyl acetate-acetate buffer†	With ethyl acetate-phosphate buffer‡	
			2:1 Na_2H-NaH_2	1:2 Na_2H-NaH_2
DNP-glycine	3	4	1	
DNP-phenylalanine	6	8	4	
2,4-Dinitroanaline	8			
2,4-Dinitrophenol	5			4
DNP-methionine	5	7	1	3
DNP-alanine	4	6	1	1
DNP-serine	3			
DNP-threonine	3	3		
DNP-proline	4, 5	6	1	1
DNP-hydroxyproline	3			
DNP-valine	7			
DNP-leucine	6			
DNP-isoleucine	6			
DNP-aspartic acid	1			
DNP-glutamic acid	1			
Di-DNP-tyrosine	8			
Di-DNP-lysine§	8	8		
ε-DNP-lysine‖	5			
DNP-arginine	5, 6			1
DNP-histidine	6, 7			
DNP-cystine	3			
DNP-glycyldehydroalanine	1**	1		
Bis-DNP-diglycyl-α-aminoalanine	4			1

* 95 cc. of water + 5 cc. of concentrated ammonium hydroxide.

† 1 volume of 0.1 M acetic acid + 3 volumes of 0.1 M sodium acetate.

‡ Buffer made by mixing 0.1 M solutions of Na_2HPO_4 and of NaH_2PO_4 in the proportions indicated.

§ In a system composed of 5 per cent aqueous ammonium hydroxide as the bottom layer and 2:1 ethyl acetate-ether as the top layer, the peak was in Tube 3. With just ethyl acetate as the top layer the peak was in Tube 6. The sodium salt was completely extractable from aqueous solution with ethyl acetate.

‖ In a system of butanol and 0.1 N HCl, the peak was in Tube 6.

** The peak was in Tube 2 when 3 volumes of butanol to 1 volume of ammonia were used instead of the 1:1 ratio.

the butanol counter-current distribution by a partition with ethyl acetate and 0.1 M phosphate buffer made from 2 volumes of Na_2HPO_4 and 1 volume of NaH_2PO_4.

(*c*) *Demonstration of DNP-Glycine in Fraction III of DNP Insulin Digest*—100 mg. of Fraction III, the part not extractable by ethyl acetate or by butanol, were hydrolyzed with HCl and separated exactly as described for DNP insulin in the preceding paragraph, and DNP-glycine was readily obtained.

(*d*) *Stability of DNP-Glycine to Hot, 20 Per Cent HCl*—5 mg. of DNP-glycine were refluxed in 15 cc. of 20 per cent HCl for 24 hours, and the reaction mixture was concentrated to dryness under reduced pressure. The residue was distributed counter-currently between butanol and dilute ammonia through eight tubes. Most color was found in Tube 3, but a considerable amount of orange color was in Tube 8 (*cf.* (2)). Thus, some decomposition had occurred during the prolonged acid treatment.

(*e*) *Investigation of Chromophoric Group of DNP Peptides Isolated from DNP Insulin Digests*—Samples of 10 to 30 mg. of each of the compounds were refluxed in 15 cc. of 20 per cent HCl for 4 hours, and the acid was removed by evaporation under reduced pressure. The residue was then taken up in water, and the suspension which resulted was extracted five times with ethyl acetate. The extract was freed of solvent and counter-currently distributed between butanol and dilute aqueous ammonia. In every case, the major part of the color was divided into two components, one of which remained mostly in Tube 1 and the other in Tube 8. In the case of the butanol mid-piece, the butanol right end-piece, and the ethyl acetate mid-piece, a faint peak was discernible in Tube 3, which should represent DNP-glycine. Therefore, the contents of Tubes 2 to 4 were freed of solvents under reduced pressure and counter-currently distributed between ethyl acetate and 0.1 M acetate buffer (made from 3 volumes of sodium acetate and 1 of acetic acid). When the distribution was continued through eight tubes, a peak characteristic for DNP-glycine appeared in Tube 4. However, the amount of color thus to be attributed to DNP-glycine was only a small fraction of the total in the hydrolysate. The major colored hydrolysis product was found in the right-hand tube of the butanol-ammonia counter-current series, and this was shown to be a neutral substance because it could be extracted from either strongly acidic or alkaline solutions into ether. Results practically identical with these were found when the period of hydrolysis was 20 hours instead of 4.

There was some evidence for another colored component in the hydrolysates because the acidic aqueous phase which remained after the ethyl acetate extraction was still brownish yellow. It was not known whether this color arose from decomposition during hydrolysis or whether it was due to some yellow constituent not extractable with ethyl acetate. It was noted particularly in hydrolysates of the mid-pieces. On a paper strip chromatogram with butanol and ammonia this color moved at the same rate as did ϵ-DNP-lysine.

(*f*) *Anomalous Behavior of Bis-DNP-diglycyl-α-aminoalanine on Acid Hydrolysis*—Diglycyl-α-aminoalanine (10) was converted to the DNP derivative according to the general procedure described by Sanger for DNP amino acids, and the bis-DNP-diglycyl-α-aminoalanine was purified by counter-current distribution between butanol and dilute aqueous ammonia (*cf.* Table III). It was finally obtained as the water-insoluble free acid which melted at 170–175°.

$C_{19}H_{18}O_{12}N_8 \cdot H_2O$. Calculated, N 19.8; found, N 19.5

10 mg. were refluxed for 4 hours in 20 per cent HCl, and the product was fractionated counter-currently with butanol and dilute ammonia. No trace of starting material or of DNP-glycine was found, but instead, two colored fractions, a left end-piece and a right end-piece, were present.

By treatment with cold, concentrated HCl, followed by separation and hydrolysis of the individual cleavage products, DNP-glycine could be isolated from the compound, thus leaving little doubt that it was actually contained in the molecule. Cold, concentrated HCl (0.5 cc.) in 4 hours split the compound (5 mg.) quantitatively into two colored substances which were separated by counter-current distribution between butanol and aqueous ammonia. One product was identified as DNP-glycyl-dehydroalanine, found in Tubes 1 and 2 of the counter-current series,[10] and the other was a neutral compound, found in Tube 8, with the melting point of DNP-glycine amide (245°). When either one of these products was refluxed alone in 20 per cent HCl, DNP-glycine was formed, which was isolated by methods already described.

(*g*) *DNP-Methionine from Hydrolysate of Ethyl Acetate Slow Piece of DNP Trypsinogen*—4 mg. of the ethyl acetate slow piece from DNP trypsinogen were dissolved in 15 cc. of 20 per cent HCl and the solution was refluxed for 24 hours. Excess HCl was removed under reduced pressure, the residue was suspended in water, and the suspension was extracted three times with ethyl acetate. The extracts were freed of solvent under reduced pressure and the yellow substance was distributed counter-currently, first in butanol and dilute aqueous ammonia, then in ethyl acetate-acetate

[10] If care was exercised to prevent the temperature from rising during any of the operations, an intermediate compound could be distinguished as a colored peak in Tube 2 of a series of eight counter-current tubes. This was probably DNP-glycyl-α-hydroxyalanine. Gentle warming such as that which occurred during concentration under reduced pressure resulted in the disappearance of this material and the formation of a compound which behaved in a counter-current distribution as did synthetic DNP-glycyldehydroalanine. The course of the hydrolysis, therefore, was the cleavage to DNP-glycine amide by reaction of 1 molecule of water, and the subsequent dehydration of the relatively unstable derivative of α-hydroxyalanine. Synthetic glycyldehydroalanine used for the preparation of the DNP derivative was kindly supplied by Dr. J. P. Greenstein.

buffer, and finally in ethyl acetate and phosphate buffer. The compositions of these buffers were those shown in Table III. The yellow material behaved in each of the solvent systems as did DNP-methionine (*cf.* Table III). The quantity of material available was not sufficient for identification by classical means. In addition to the yellow substance which behaved as DNP-methionine, there was also some orange material which remained in the right-hand end tube of the butanol-ammonia distribution.

Partial Hydrolysis with Cold, Concentrated HCl of Slow Moving Butanol Left End-Piece—25 mg. of the slow moving butanol left end-piece were dissolved in 5 cc. of concentrated HCl for 5 hours at room temperature and the solution was then diluted with 25 cc. of water and extracted twice with butanol. The extracts were treated with 10 cc. of dilute aqueous ammonia, concentrated under reduced pressure to dryness, and the residue was distributed counter-currently between butanol and dilute aqueous ammonia through a series of eight tubes. Peaks of color appeared in Tubes 1, 3, and 8. Contents of Tubes 2, 3, and 4 were freed of solvents under reduced pressure and the residue was distributed counter-currently between butanol and dilute aqueous ammonia through twelve tubes. 7.5 cc. of butanol and 2.5 cc. of aqueous phase were used in each tube. The compound which was now found in Tubes 3, 4, and 5 was obtained as a small amount of crystalline material by evaporation of the solvents. Hydrolysis and analysis showed the presence of only glutamic acid, leucine, and isoleucine.

Partial Hydrolysis with Cold, Concentrated HCl of Butanol Mid-Piece—10 mg. of the butanol mid-piece from DNP insulin were treated with HCl in the manner described in the preceding section. In the counter-current separation, the major part of the color appeared as a peak in Tube 8 of a series of fourteen. When the solutions containing this colored material were evaporated and the residue hydrolyzed, glutamic acid was found as the major amino acid. Traces of the other amino acids derivable from the butanol mid-piece could be detected if relatively high concentrations of the hydrolysate were tested. Because the cleavage product appeared in the same position in a counter-current distribution as did the butanol mid-piece from which it was made, this contamination was impossible to avoid. A similar cleavage product of the butanol mid-piece of DNP trypsinogen was found.

SUMMARY

Methods have been described for the separation of dinitrophenyl amino acids (DNP amino acids) and of DNP peptides. These methods depend on differential extraction and on counter-current distribution of various salts of the DNP compounds.

Pancreatin digests of DNP insulin have been fractionated by these methods to yield four crystalline yellow compounds. These same four crystalline compounds were also obtained from pancreatin digests of the DNP derivative of that fraction of performic acid-oxidized insulin which was soluble at pH 6. Evidence for the homogeneity of these products was presented.

Two compounds indistinguishable in all respects from two of those from DNP insulin were also isolated from digests of DNP trypsinogen. In addition a third substance, not obtainable from DNP insulin, was isolated from DNP trypsinogen and found to yield DNP-methionine, serine, and isoleucine when it was hydrolyzed.

The amino acid composition of each of the substances from DNP insulin was examined, and they were found to be of differing complexity. One contained the chromophoric group and glutamic acid; the second yielded the chromophoric group, glutamic acid, serine, threonine, alanine, valine, leucine, and isoleucine; a third gave these constituents plus aspartic acid; and a fourth contained the same amino acids (except alanine) as the third but differed from it in the order of their arrangement. A fifth substance, the purity of which was not established, was found to contain the chromophoric group, aspartic acid, glutamic acid, serine, alanine, valine, leucine, isoleucine, and cystine. The regular increase in complexity suggested that the simpler ones were degradation products of the more elaborate ones. Some evidence against this view was presented. Although some of the smaller peptides could be derived from one of the largest, another one of the largest molecules apparently had its amino acid residues arranged differently. The amino acid residues occurred in these compounds in equimolecular proportions, except for one in which approximately 3 moles of leucine were present per mole of other constituents.

The chromophoric group was considered to be DNP-glycine, but because of the low yield of this substance which could be isolated from the hydrolysates of the peptides, there was much doubt about this conclusion. Most of the colored material, or chromophore which was liberated by acid hydrolysis, could not be identified with previously described yellow products from DNP insulin. A model compound, bis-DNP-diglycyl-α-aminoalanine, was synthesized and found to behave on hydrolysis with respect to its chromophoric groups much as did the peptides from insulin.

Partial hydrolysis of the larger of the DNP peptides gave rise to smaller yellow compounds and these were isolated. From one of the large peptides the cleavage product was shown to contain the chromophore, leucine, isoleucine, and glutamic acid. From another of the larger peptides the partial cleavage product was composed principally of the chromophore and glutamic acid.

BIBLIOGRAPHY

1. Woolley, D. W., *J. Biol. Chem.*, **171,** 443 (1947).
2. Sanger, F., *Biochem. J.*, **39,** 507 (1945).
3. Woolley, D. W., *J. Biol. Chem.*, **172,** 71 (1948).
4. Sanger, F., *Nature*, **160,** 295 (1947).
5. Craig, L. C., Golumbic, C., Mighton, H., and Titus, E., *J. Biol. Chem.*, **161,** 321 (1945).
6. Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, **38,** 224 (1944).
7. Snell, E. E., *Ann. New York Acad. Sc.*, **47,** 161 (1946).
8. Hac, L. R., and Snell, E. E., *J. Biol. Chem.*, **159,** 291 (1945).
9. Hac, L. R., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, **159,** 273 (1945).
10. Gonçalves, J. M., and Greenstein, J. P., *Arch. Biochem.*, **16,** 1 (1948).
11. Woolley, D. W., *Federation Proc.*, **7,** 200 (1948).

[Reprinted from the JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY, July, 1949, Vol. 33, suppl., pp. 1–231]

ON THE EFFECT OF CERTAIN QUATERNARY AMMONIUM IONS UPON FROG NERVE

PARTS I AND II

BY RAFAEL LORENTE DE NÓ

(From the Laboratories of The Rockefeller Institute for Medical Research)

CONTENTS

PART I

ON THE EFFECT OF CERTAIN QUATERNARY AMMONIUM IONS UPON FROG NERVE

Part I

By RAFAEL LORENTE DE NÓ

Fifty-Two Figures

1. INTRODUCTION

As originally demonstrated by Overton ('02) and repeatedly confirmed by the present writer (Lorente de Nó, '44, '47)[1] frog nerve becomes inexcitable after it has been kept in a sodium-free medium for a certain number of hours; in addition, important changes take place in the properties of the membrane of the nerve fibers ('47, sections VI.11, VII.6, VIII.4 and XV.7). If the absence of sodium is not prolonged beyond a certain duration, both the inexcitability and the changes in the properties of the membrane are reversible, restoration taking place after Na^+ ions are made available to the nerve. Overton used sugar solutions as sodium-free media; the present writer found that sugar solutions have certain effects upon the membrane potential of nerve ('47, Chapter IV) and therefore preferred the use of solutions of several quaternary ammonium bases, in which the nerve fibers maintain their membrane potential at a practically normal level. The majority of the experiments were done with the use of solutions of choline chloride, but two other quaternary ammonium ions were found to have practically no effect upon the total value of the resting membrane potential, acetylcholine[2] and β-methylacetylcholine (mecholyl).

Two conditions must be fulfilled in the preparation of a sodium-free medium suitable for the analysis of the effect of the lack of sodium upon the excitability of the nerve fibers: (1), the solution must be approximately isosmotic with Ringer's solution (0.11 M) and (2), it must not cause a significant depolarization of the nerve fibers. The first condition is not critical, since even relatively large changes in the osmotic pressure of the external medium have but little effect upon the properties of the nerve fibers (cf. below, section 4,a); the second condition, however, is essential, since a depolarization of the nerve fibers below the critical excitability level by itself is sufficient to cause inexcitability ('47, section I.7 and Chapter XIII). The chlorides of the ordinary monovalent ions

[1] Henceforth, the author's name will be omitted in the references to this study and to other papers by the present writer, except in the case of papers written in collaboration.

[2] Acetylcholine can be used to prepare a sodium-free solution only in the presence of an esterase inhibitor. In the absence of an inhibitor acetylcholine at the 0.11 M concentration causes a progressive depolarization of the nerve fibers, which in all probability is referable to the action of the acetic acid produced by hydrolysis of the substance (cf. '47, section IV.7).

(K^+, Rb^+, Cs^+, Li^+ and NH_4^+) cannot be used to prepare sodium-free solutions because at the 0.11 M concentration those ions are strongly depolarizing agents *(cf. Gallego and Lorente de Nó, '47). On the other hand, it cannot be assumed that all quaternary ammonium ions would serve to prepare sodium-free solutions, since at the 0.11 M concentration thiamine chloride causes a far reaching depolarization of the nerve fibers (47, section IV.6). The properties of the individual quaternary ammonium ions must be analyzed experimentally.*

Experiment has revealed that nerves kept in a 0.11 M solution of either tetramethyl-ammonium chloride or tetraethyl-ammonium chloride maintain the total value of their membrane potential at nearly the normal level. Thus, tetramethyl-ammonium and tetraethyl-ammonium resemble choline insofar as they do not cause a progressive depolarization of the nerve fibers. In regard to the excitability the situation is different. Nerves kept in 0.11 M tetramethyl-ammonium chloride become inexcitable in about the same manner as nerves kept in a solution of choline chloride. In nerves kept in 0.11 M tetraethyl-ammonium chloride only the fibers of fast conduction become inexcitable; a large number of fibers of slow conduction retain their ability to conduct impulses practically as long as they would in Ringer's solution. Moreover, if after a nerve has become inexcitable in 0.11 M tetramethyl-ammonium chloride or choline chloride, tetraethyl-ammonium ions are made available to it a large number of fibers of slow conduction rapidly regain their excitability.

Whichever the detailed explanation of the phenomenon may ultimately prove to be, the fact that the fibers of fast conduction and the fibers of slow conduction behave differently in the presence of tetraethyl-ammonium ions indicates that certain chemical properties of these two types of fibers are different. For this reason it is advisable to divide the fibers of frog sciatic nerve into two classes, A and Et. The fibers of class A are those which lose their excitability in 0.11 M tetraethyl-ammonium chloride and the fibers of class Et those which remain excitable. The A class corresponds to the A group in Erlanger and Gasser's classification (cf. Erlanger, '37), while the fibers of class Et belong to the B and C groups in Erlanger and Gasser's classification. The need of using the new denomination arises from the circumstance that the available evidence is not sufficient to decide whether or not all the fibers of the B and C groups belong to the Et class (cf. below, section 10,c). If future research should demonstrate that all the B and C fibers belong to the Et class, which is not unlikely, then there would be no need of using the new denomination Et. At times, it will be convenient to refer to the A fibers as the fibers of fast conduction and to the Et fibers as the fibers of slow conduction.

The sharp contrast between the effects upon nerve of tetramethyl-ammonium and choline on one hand and of tetraethyl-ammonium on the other can be explained solely in terms of changes in the properties of quaternary ammonium ions which result from the substitution of ethyl groups for methyl or for methyl

and ethanol groups. Therefore, it became imperative to analyze systematically the effects upon nerve of all those quaternary ammonium ions that contain different combinations of methyl (-CH_3), ethyl (-CH_2-CH_3) and ethanol (-CH_2-CH_2OH) groups.

The table which is presented in figure 1 gives the chemical formulae of the 15 quaternary ammonium ions that contain combinations of methyl, ethyl and

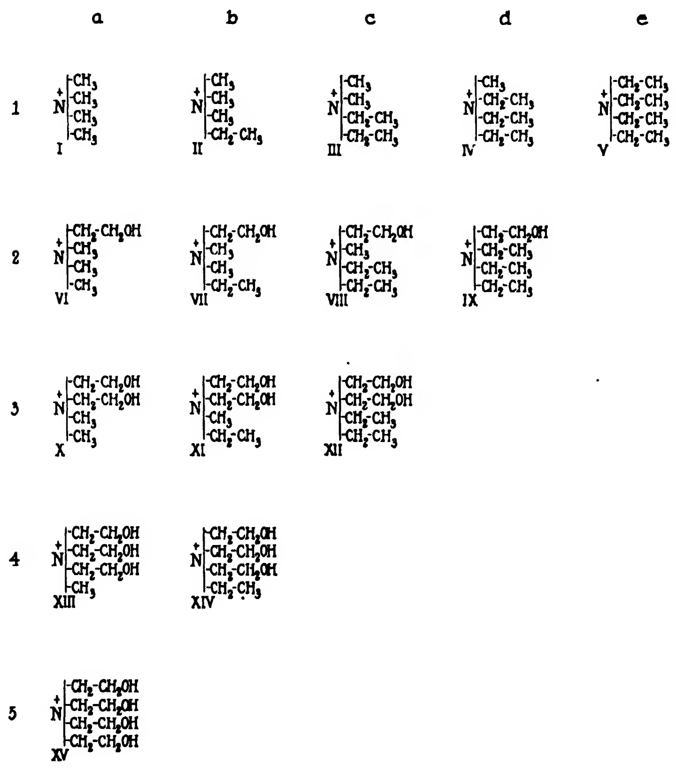


FIG. 1. The 15 possible combinations of methyl (-CH_3), ethyl (-CH_2-CH_3) and ethanol (-CH_2-CH_2OH) groups in quaternary ammonium ions. The Roman numerals I to XV are used in the text to identify the individual ions.

ethanol groups. Methods for the preparation of those compounds have long been known (Hofmann, 1851; Wurtz, 1868), and in the past several of the ions listed in figure 1 have been used by numerous investigators in pharmacological studies; indeed, the literature on the pharmacology of quaternary ammonium bases is exceedingly extensive. The excellent monograph by Guggenheim ('40) contains systematic presentations of work done along a number of lines of

research; more recent publications are listed in a paper by Acheson and Moe ('46). Particular mention should be made here of information available on

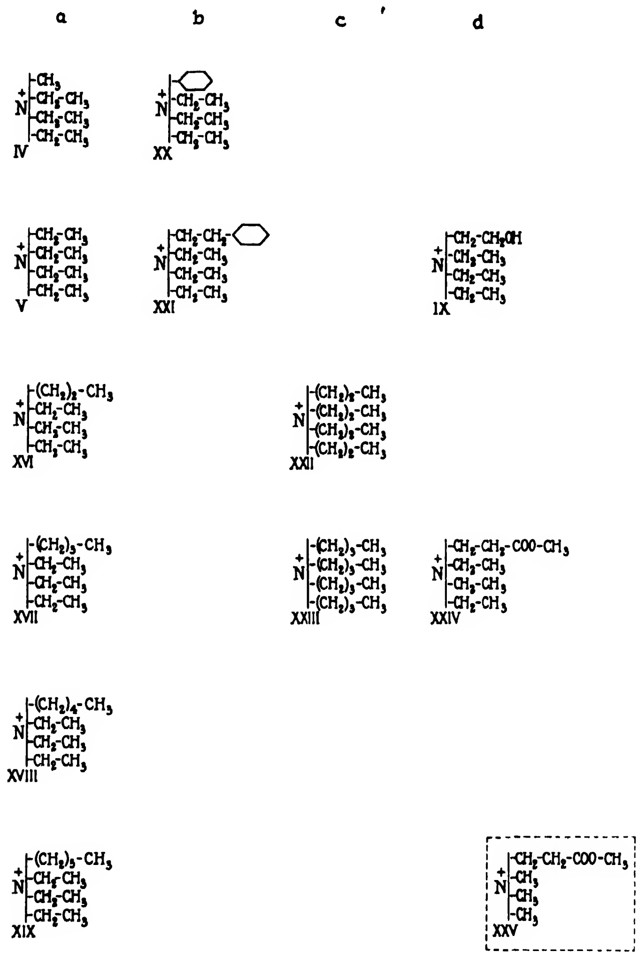


FIG. 2. Quaternary ammonium ions of the restoring type. The ions in columns a, b and d have three ethyl groups, those in column c have no ethyl groups. The formula of the acetylcholine ion (XXV) is included solely for the purposes of comparison, since acetylcholine belongs to the inert group.

changes in the pharmacological action of tetramethyl-ammonium that results from replacing one or more methyl groups by other alkyl groups. The literature has been summarized by Ing ('36); Raventós ('37); and Alles and Knoefel ('39).

Also mention should be made of observations on the effect of tetraethyl-ammonium upon nerve fibers that have been recently described by Brink, Bronk and Larrabee ('46).

The table in figure 1 has been prepared according to the following system. All the ions on each column contain the same number of ethyl groups, and all the ions in each row contain the same number of ethanol groups; the number of ethyl groups increases from column a to column e, and the number of ethanol groups increases from row 1 to row 5.

Experiments have been done with the use of all the ions listed in figure 1. One of the results obtained may be mentioned here. After a nerve has become inexcitable in a sodium-free medium, for example in a 0.22 M solution of saccharose or in a 0.11 M solution of ion I or II, the excitability of fibers of class Et can be restored by making available to the nerve one of the ions listed in columns c, d, and e of figure 1, i.e., one of the ions that contains two or more ethyl groups. Not all the restoring ions, however, are equally effective; the effectiveness, as measured both by the rapidity of action and by the number of restored fibers, increases with the number of ethanol groups and more so with the number of ethyl groups present in the ion. Consequently, among the 7 ions listed in column c, d and e of figure 1, tetraethyl-ammonium is the most effective restoring agent.

This observation created the need of further investigation along two different lines. In the first place, since tetraethyl-ammonium is not able to restore the excitability of A fibers deprived of sodium, it seemed necessary to investigate whether or not the excitability of A fibers would be restored by ions having, in addition to three ethyl groups, a different group. The ions used in this investigation are listed in columns a, b and d of figure 2; restoration of the excitability of A fibers is not effected by any of the ions used, even though all of them are able to restore the excitability of Et fibers. On the other hand, it seemed necessary to investigate whether or not the ethyl group plays a specific rôle in the restoration of the excitability of Et fibers. A definite answer to this question was obtained. Restoration is effected by the two ions listed in column c of figure 2, which have no ethyl groups. Consequently, the ethyl group does not play a specific rôle in the restoration process.

2. *Technique*

a. Preparative. From the compounds listed in figure 1 only three (I, V, VI) are commercially available in chemically pure form (Eastman Chemicals, tetramethyl-ammonium hydroxide, tetraethyl-ammonium bromide, choline chloride). The other compounds were prepared from Eastman Chemicals by the use of the methods described by Hofmann (1851) and Wurtz (1868). The reactions used to prepare the individual compounds were the following: II trimethylamine and ethyl iodide; III, diethylamine and methyl iodide; IV,

triethylamine and methyl iodide; VII, dimethylaminoethyl alcohol and ethyl iodide; VIII, diethylaminoethyl alcohol and methyl iodide; IX, diethylaminoethyl alcohol and ethyl iodide or triethylamine and ethylene chlorohydrin; X, dimethylaminoethyl alcohol and ethylene chlorohydrin; XI, ethyldiethanolamine and methyl iodide; XII, diethylaminoethyl alcohol and ethylene chlorohydrin; XIII, triethanolamine and methyl iodide; XIV, triethanolamine and ethyl iodide; XV, triethanolamine and ethylene chlorohydrin.

From the compounds listed in figure 2 only three, XXII, XXIII and XXV, are commercially available (Eastman Chemicals, tetra-n-propyl-ammonium iodide, tetra-n-butyl-ammonium iodide; Merck, acetylcholine chloride). The other compounds were prepared by the use of the following reactions: XVI to XIX, triethylamine and the corresponding alkyl iodide; XX, diethylaniline and ethyl iodide; XXI, triethylamine and β-phenylethyl bromide. Compound XXIV was prepared by acetylation of compound IX with acetic anhydride in the presence of acetic acid.

The purification of the compounds did not offer any difficulty. As a rule crystallization of the iodide or of the chloride from absolute alcohol was sufficient; in some cases, in which this procedure failed to give satisfactory results, the quaternary ammonium ions were precipitated from an alcohol solution of their chloride as complex mercury salts; the precipitate was suspended in water, the mercury removed with hydrogen sulfide and the solution neutralized with silver oxide.

Although the presence of a significant amount of sodium in any of the prepared compounds was exceedingly unlikely, the absence of sodium was proven in every instance by means of zinc uranyl acetate, that detects the presence of sodium in concentrations far below those which are significant in experiments on nerve function.

All the experiments have been done with the use of the chlorides of the quaternary ammonium ions. Choline chloride has been used at two concentrations, 0.11 M and 0.18 M (cf. section 4,a); all the other chlorides, at the 0.11 M concentration. In the majority of the experiments K^+ ions were added to the solution at the concentration of 0.003 M, the corresponding anions being two phosphate ions in such a proportion that the pH of the solution was 7.3.

b. Experimental. The effect of quaternary ammonium ions upon frog nerve has been analyzed in two parallel series of experiments. In the experiments of one series measurements were made of the changes in the resting membrane potential; in the experiments of the other series oscillographic analysis was made of the changes in the polarizability of the membrane and in the ability to conduct impulses. As a rule, the nerves were kept in an atmosphere of air.

The technique used to measure changes in the resting membrane potential has been described elsewhere ('47, section I.2). In principle the method consists in measuring at properly selected intervals of time the difference of potential

established between a segment of nerve (A) in contact with Ringer's solution and a segment (B) in contact with the test solution (cf. diagram in fig. 38). The length of the B segment was 20 mm and the distance between the neighboring margins of the A and B vessels, i.e., the length of the AB segment, 26 mm.

The experiments done with the use of oscillographic technique were carried out in the following manner. Immediately after excision the nerve was placed in a relatively very large volume (50 ml) of the sodium-free solution of the chloride of a quaternary ammonium ion. The solution was renewed usually 4, never less than three times at 30-minute intervals; after the last change the solution could not be expected to contain any other sodium ions than those which would slowly diffuse outward from the nerve fibers themselves, and since the volume of the solution was very large in relation to that of the nerve the concentration of sodium outside the nerve fibers had to be negligible at all times. As a matter of fact, after the last change, zinc uranyl acetate always failed to detect the presence of sodium in the test solution. The nerve was mounted in a moist chamber after it had been in the test solution for a certain number of hours, usually 15 to 24. The rest of the experiment included several steps.

1. The first step consisted in the analysis with the arrangement of electrodes indicated in figure 3,I of the electrotonic potentials produced in the peripheral segment of the nerve (fig. 3, II,mr_2) by rectangular pulses of applied currents. Since the tests were made with relatively large currents, 8, 15, and 40 μa,[3] the existence of excitable fibers was readily detected; if the number of excitable fibers was large the nerve was returned to the test solution, to be mounted again in the moist chamber several hours later. If the inexcitability of the nerve was total or if it had reached the desired stage the experiment was continued.

2. The central segment of the nerve (fig. 3, II, mp_2) was placed in contact with Ringer's solution and the progress of the recovery was followed by means of frequent tests of the ability of the nerve fibers to conduct impulses, which were done with the arrangement of electrodes indicated in figure 3, II. In order to insure the contact of the nerve with an effectively large volume of Ringer's solution two thin strips of filter paper were placed alongside the central segment of the nerve and drops of Ringer's solution were deposited upon the strips at short intervals of time. It hardly need be mentioned that the strips were removed during each oscillographic test. Particular care was exerted to prevent Ringer's solution from spreading into the peripheral segment of the nerve; for this purpose the peripheral segment was maintained at a slightly higher level than the central segment. Usually one hour was allowed for the recovery of the central segment.

[3] These values, 8, 15, and 40 μa are only approximate. The currents were actually measured in the first experiment of the series only; in the other experiments the magnitude of the currents was determined by using the same settings on the dials of the square pulse generator that were used in the first experiment.

3. After the state of Ringer-treated, central segment had improved so far that all the fibers of slow conduction and at least a large majority of the fibers of fast conduction had recovered their ability to conduct impulses, tests were done of the ability of impulses initiated in the central segment of the nerve to

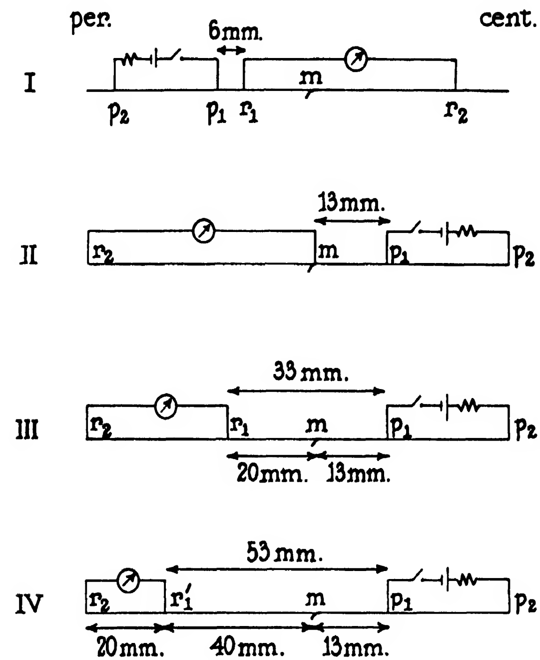


FIG. 3. Diagram of the arrangement of electrodes used for oscillographic analysis of the properties of the nerve. The point of origin of the branches for the gastrocnemius and neighboring muscles has been chosen for an arbitrary division of the sciatic nerve into central and peripheral segments; the point itself is called m (margin); p_1, p_2 electrodes of the polarizing or stimulating circuit; m, r_1, r'_1, r_2 electrodes of the recording circuit. Arrangement I has been used to analyze the electrotonic potential before and after treatment of the nerve with restoring solutions; arrangements II to IV, to analyze the ability of the nerve fibers to conduct impulses. In a few experiments the interelectrode distances were varied by 1 or 2 mm.

propagate themselves into the peripheral segment. The arrangement of electrodes routinely used in this part of the experiment is indicated in figure 3, III; in some cases also a greater conduction distance was used (fig. 3, IV).

The peripheral segment of the nerve was then placed in contact with a new test solution, i.e., a 0.11 M solution of another quaternary ammonium ion. The new test solution was applied by means of thin strips of filter paper upon

which drops of the solution were deposited at short intervals of time. The strips were removed, of course, during each oscillographic test.

4. After the new test solution had acted upon the peripheral segment of the nerve for a sufficiently long period of time or when the state of the peripheral segment, as defined by the ability of the nerve fibers to conduct impulses, made the analysis desirable, the electrotonic potentials produced by applied currents were again recorded with the arrangement of electrodes indicated in figure 3, I.

In a few instances the experiment was discontinued after step 4; usually, however, after step 4 a new test solution was applied to the peripheral segment of the nerve, and steps 3 and 4 were repeated. Often Ringer's solution was used as a third test solution, in which case steps 3 and 4 were repeated once more.

All the experiments were done with the two sciatic nerves of a bullfrog (R. catesbiana or R. gryllio). In a number of experiments the two nerves were examined in succession, but in other experiments it was found advisable to examine the two nerves simultaneously. For this purpose the nerves were mounted in two identical chambers and the peripheral segments of the two nerves were placed in contact with the new test solutions (step 3) practically at the same time.

It might be useful to mention here that the various fibers of the nerve lose their excitability in a definite order when the nerve is kept in a sodium-free medium (a 0.22 M solution of saccharose or a 0.11 M solution of one of the ions listed in figure 1 above the diagonal 4,d). The fibers that lose their ability to conduct impulses in the shortest interval of time are fibers of the A group and the fibers that remain able to conduct impulses for the longest interval of time are fibers of the C group; there are, however, fibers of the A group that are more resistant to the effect of the lack of Na^+ ions than the least resistant fibers of the B and C groups, so that the B and C elevations of the compound spike begin to decrease before the A elevation has disappeared entirely. Nevertheless, during the development of the inexcitability of the nerve a stage is reached in which no A fiber is able to conduct impulses while many fibers of slow conduction, B as well as C fibers, are able to conduct. On the other hand, as will be shown in section 4,b, at a time when all the A fibers have become unable to conduct impulses, some A fibers are still able to produce impulses in response to the opening of the anodal current, with the noteworthy peculiarity that these A fibers may be able to produce impulses at a time when all the B and C fibers are unable to conduct impulses. To avoid misunderstandings attention will be called to the difference that exists between the ability of a nerve fiber to conduct impulses in the absence of an externally applied restoring current, and the ability of an inexcitable fiber to produce impulses after its excitability has been restored by an applied anodal current (cf. '47, Chapter XIII).

Under conditions such as these it is clear that the length of time necessary to render a nerve "inexcitable" in an inert sodium-free medium depends upon

the manner in which inexcitability is defined and observed. As a rule 8 to 10 hours are sufficient to render the majority or even the totality of A fibers unable to conduct impulses, but inexcitability of all the C fibers should not be expected within less than 14–16 hours. Furthermore, considerable differences have been observed in the behaviors of individual nerves. In part the differences seem to depend upon the season of the year; inexcitability develops earlier in the warm than in the cold seasons of the year. (The experiments done with oscillographic technique have always been carried out at the same temperature, 20–22°C.). In part the individual differences seem to depend upon the state of nutrition of the frog, since considerable differences have been observed occasionally between nerves taken from frogs of the same lot.

3. *Effects of Increasing the Number of Ethyl Groups in Quaternary Ammonium Ions*

The quaternary ammonium ions used in the experiments to be described in this section contain only methyl ($-CH_3$), ethyl ($-CH_2-CH_3$) and ethanol ($-CH_2-CH_2OH$) groups. Immediately after excision the nerves were placed in a 0.11 M solution of ion I (tetramethyl-ammonium) or II (ethyl-trimethyl-ammonium) and were kept in the solution for several hours after all the nerve fibers had lost their ability to conduct impulses. Restoration of the nerve was effected by means of 0.11 M solutions of quaternary ammonium ions that contain two or more ethyl groups (fig. 1, columns c, d and e).

a. Restoration of nerve fibers by tetraethyl-ammonium. Figures 4 and 5 illustrate the results of a typical experiment. The observations were begun after the nerve had been in a 0.11 M solution of tetramethyl-ammonium chloride for 29 hours.

Records *1* to *8* of figure 4 present the electrotonic potentials that were produced by rectangular pulses of applied current (8 and 15 μa) at 6 mm from the polarizing electrode. In the main, therefore, records *1* to *8* of figure 4 characterize the state of the fibers of the A group (cf. section 9,a).

In the cathodal records (*1,5*) i.e., in those which were obtained with the use of applied currents of which electrode p_1 was the cathode, there appears only the fast electrotonus (cf. '47, section VI.3), in the form of nearly perfect, rectangular deflections. Since similar records were also obtained with the use of smaller currents the lack of a slow component in the catelectrotonus can be taken as a proof that the L fraction of the membrane potential had a negligible value.[4] This conclusion is in agreement with the behavior of the anelectro-

[4] The value of the L fraction of the membrane potential is measured by the height of the slow catelectrotonus produced by currents of moderate magnitude and relatively short duration. With excitable nerve currents approximately equal to twice the rheobase of the most irritable fibers and of about 1 second duration create the most favorable conditions for the measurement of the L fraction (cf. '47, section III.8). With nerves that are inexcitable, the identification of the L fraction and the slow cate-

tonus (records *2* to *4*, *6* to *8*). The low rate of increase of the slow anelectrotonus and the lack of maxima during the 10-second pulses used to obtain records *3*, *4* and *7*, *8* are proof of the low polarizability of the boundary at which the slow electrotonus is established by the applied current and of the lack of effectiveness of the nerve reaction (E_3 reaction). As described elsewhere ('47, Chapter VII) the polarizability of the membrane and the value of the L fraction of the membrane potential vary in parallel manners, or otherwise stated, a low polarizability of the boundary at which the L fraction is maintained indicates that the value of the L fraction is small (cf. below, section 9).

Under conditions such as these one should expect that the membrane potential of frog nerve kept in tetramethyl-ammonium would undergo a significant decrease. In contrast with this expectation experiment has shown that nerves kept in 0.11 M tetramethyl-ammonium chloride maintain the total value of their membrane potential at practically the normal level (cf. below, section 8). Therefore, the state of nerves kept in tetramethyl-ammonium is comparable with the state of nerves at low temperature, in which the total value of the membrane potential has very nearly the normal value, but the L fraction is exceedingly small (cf. '47, section VIII.3a).

Tetraethyl-ammonium does not restore the excitability of the fibers of the A group; nevertheless, it is able to produce a marked improvement of the properties of the membrane of those fibers. Records *9* to *16* of figure 4 were obtained after the nerve had been in contact with a 0.11 M solution of tetraethyl-ammonium chloride for slightly over one hour. The improvement of the state of the nerve is noticeable in the cathodal records (*9*, *13*) in that the slow electrotonus displayed slow components, which was a proof that tetraethyl-ammonium had been able to produce a significant increase in the L fraction of the membrane potential. The anodal records show that the polarizability of the membrane had undergone a marked increase, since the slow anelectrotonus was established by the current at a higher rate than previously (cf. records *2* and *10*, *6* and *14*). In addition, the slow anelectrotonus displayed definite maxima during the flow of the applied current (records *11*, *12*; *15*, *16*) and overshootings after the end of the polarization (records *10*, *12*; *14*, *16*). Qualitatively, the effect of tetraethyl-ammonium was exceedingly similar to the effect that would have been produced by sodium (cf. below).

Figure 5 illustrated the effects of sodium upon the excitability of the central segment of the nerve (records *1* to *4*) and of tetraethyl-ammonium upon the excitability of the peripheral segment (records *9* to *19*). The central segment of

---

lectrotonus presents certain theoretical difficulties. This question, however, has no important bearing on the problems analyzed in the present paper. For simplicity the slow catelectrotonus produced by brief pulses of current will be regarded as a true measure of the L fraction; it would be only in the case of long lasting polarization that this procedure could lead to significant error.

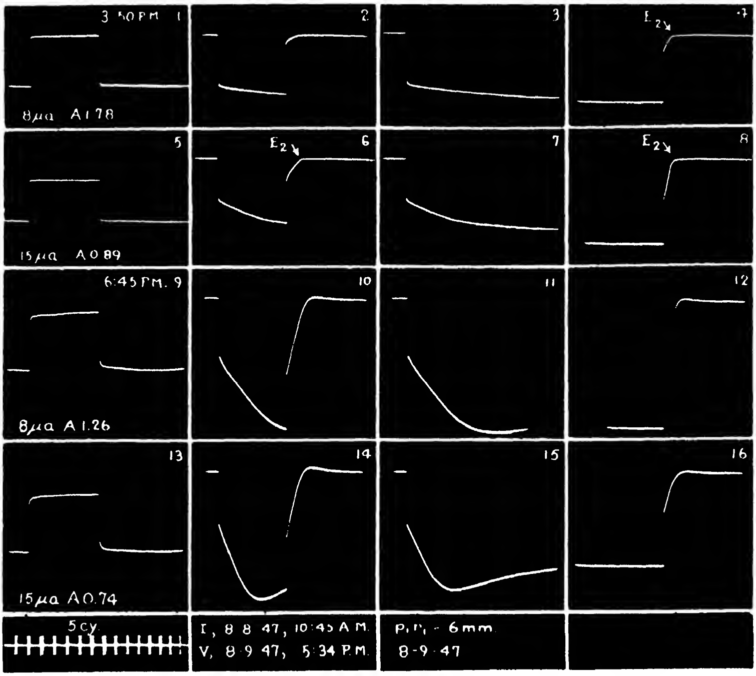
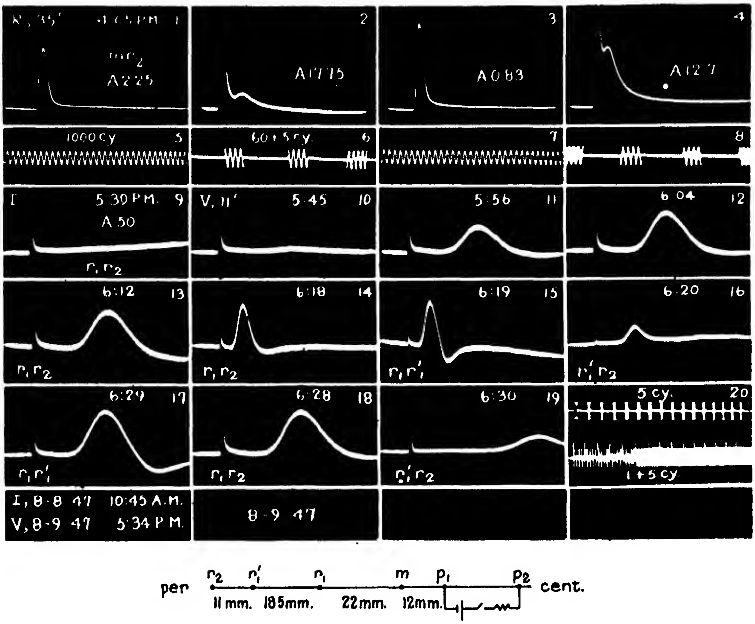


FIG. 4. Electrotonic potentials produced by rectangular pulses of current at 6 mm from the polarizing electrode (fig. 3, I) in a nerve kept in a 0.11 M solution of tetramethyl-ammonium chloride (fig. 1, I) for 29 hours (records *1* to *8*) and restored by 0.11 M tetraethyl-ammonium chloride (records *9* to *16*). The arrows labelled E_2 mark sharp changes in the slope of the tracing of the slow anelectrotonus, which correspond to the E_2 overshootings of the slow anelectrotonus of normal nerves.

In this and all similar figures the records of the last two columns were obtained with the use of 10-second pulses of current. The zero potential level is common to the three anodal records of each horizontal row. The magnitude of the applied current and the amplification used are indicated, as a rule, with the records of the first column; the amplification (A) is given in mm deflection per millivolt input, when the distance between the two vertical lines enclosing each column of records measures 31.5 mm. The ions used to treat the nerve are identified by the Roman numerals that appear in figures 1 and 2. The times at which the solutions came in contact with the peripheral segment of the nerve are given, as a rule, at the bottom of the figures. The times at which the observations were made are given with the individual records.

FIG. 5. Restoration of the excitability of nerve fibers deprived of sodium in a 0.11 M solution of tetramethyl-ammonium chloride (fig. 1, I). The diagram at the bottom indicates the interelectrode distances used. The central segment was treated with Ringer's solution (R.); the peripheral segment with a 0.11 M solution of tetraethyl-ammonium chloride (V); no restoring solution was applied to the r'_1r_2 segment.

1 to *4*, restoration of the central segment. *1* and *3* spikes of fibers of fast conduction; *2* and *4*, spikes of fibers of slow conduction. Record *3* was obtained at 5:26 P.M. The time lines *5* to *8* apply to the corresponding records of the series *1* to *4*. In this and



similar figures a row of time lines is used to separate observations made with the central segment from observations made with the peripheral segment of the nerve. *9* to *19*, restoration of the peripheral segment. The lower time line in record *20* applies to records *14* to *16*, the upper line to the other records of the series *9* to *19*. *9*, lack of conducted response before the application of the restoring solution. Note the upward drift of the base line; similar drifts appear in other records (*13*, *15*, *16*) as well as in records of other figures. The drifts were referable to slow variations in the potential of the recording, silver-silver chloride electrodes; for this reason they were greatest immediately after each change in the solution in contact with the nerve; even when they were great, they did not interfere with the observation of conducted spikes.

In this and all the similar figures the composition of the solution in contact with the nerve is given on the upper left corner of the first record of each series (cf. records *1*, *9*, *10*); the Roman numerals identify the ions listed in figures 1 and 2; R. indicates Ringer's solution. Usually, the interval of time elapsed since a new test solution was applied is also given; for example V, 11′ in record *10* indicates that the 0.11 M solution of tetraethyl-ammonium chloride had been placed in contact with the nerve 11 minutes before record *10* was obtained. The time at which the records were obtained is given with the records; when no time is given either with the record or in the legend of the figure the record was obtained shortly after the preceding one. When the interval of time elapsed between successive records is significant it is given in seconds on the upper left corner of the records. For example, record *21* of figure 6 was obtained at 8:30 P.M., record *22*, 10 seconds after *21*, record *23*, 10 seconds after *22* and record *24*, 10 seconds after *23*. The amplification is given with the first record obtained at a new amplification. The arrangement of electrodes used or the conduction distance is also given.

the nerve was placed in contact with Ringer's solution, i.e., practically with 0.1 M sodium chloride at 3:30 P.M., and records *1* to *4* were obtained at the indicated times. The recovery of excitability was not yet complete after 35 minutes of the action of sodium (records *1*, *2*), but it became practically complete in the following 20 minutes (records *3*, *4*). It should be noted that the recovery of the fibers of slow conduction began earlier and progressed at a higher rate than the recovery of the fibers of fast conduction (cf. below, section 10,c). For the purpose of the present discussion it is important to mention that fibers of slow conduction began to become excitable about 7–8 minutes after Na^+ ions had been made available to them and that, as already mentioned, the recovery needed more than 35 minutes for completion.

Record *9* shows that after the central segment of the nerve had performed its recovery no fiber was able to conduct impulses into the peripheral segment. This segment was then placed in contact with a 0.11 M solution of tetraethyl-ammonium chloride, which was followed by a prompt recovery of the ability of fibers of class Et to conduct impulses. A conducted spike appears in record *10* that was obtained after 11 minutes of the action of tetraethyl-ammonium ions. The progressive growth of the number of conducting fibers is placed in evidence by records *11*, *12*, *13*, and *18* that were obtained respectively 22, 30, 38 and 54 minutes after tetraethyl-ammonium ions had been made available to the nerve. Comparison of records *12* and *18* shows that the restoration was approaching its maximum after 30 minutes of the action of tetraethyl-ammonium. Thus, there can be no doubt that the action of this ion is practically as fast as that of sodium. It is true that tetraethyl-ammonium restores the ability to conduct impulses only to a certain class of fibers, but the recovery of these fibers is initiated and completed as fast by tetraethyl-ammonium ions as by sodium ions.

Three important differences exist between the properties of nerve fibers restored by sodium and of fibers restored by tetraethyl-ammonium. In the first place, nerve fibers restored by tetraethyl-ammonium conduct impulses at a greatly reduced speed. A rather accurate evaluation of the speed of conduction can be made by measuring the difference between the shock spike times in records *18* and *19* of figure 5. The difference measures approximately 750 msec; since the difference between the conduction distances was 18.5 mm, it follows that the fastest among the restored fibers were conducting impulses at the speed of 25 millimeters (*sic*) per second. It will be shown below (section 10,c) that among the restored fibers there were some that under normal conditions conduct impulses at a rate of no less than 1 m per second. If, for the sake of the argument, the rate of 1 m per second is regarded as accurate, it follows that after restoration by tetraethyl-ammonium the speed of conduction is $\frac{1}{40}$ of the normal speed. To be sure, the speed of conduction of fibers restored by tetraethyl-ammonium may vary between rather wide limits depending upon

the length of time during which the nerve fibers have been deprived of sodium; it is also true that the speed of conduction of fibers restored by Na^+ ions is quite low during the initial stages of the recovery (cf. below, section 10,b and c); all these circumstances, however, do not modify essentially the fact, that fibers restored by tetraethyl-ammonium ions always conduct at a tremendously reduced speed.

The second difference between nerve fibers restored by sodium and fibers restored by tetraethyl-ammonium is that the spike, or to be more specific, the ascending as well as the descending leg of the spike, has a greater duration in fibers restored by tetraethyl-ammonium.

The problem of the relationship of the recorded external spike to the membrane action potential, which is already very complex in the case of fibers of fast conduction (cf. '47, section V.6 and XV.6), presents in the case of fibers of slow conduction additional difficulties, one of which is that the shape of the spike varies between exceedingly wide limits depending upon the experimental conditions, while the presence of A fibers in the nerve makes it impossible to use applied currents to analyze the spike of the fibers of slow conduction by means of those methods, which proved to be so valuable in the study of the A spike itself (cf. '47, Chapter XIV). An illustration of the difficulties encountered in the analysis of the Et spike might prove to be useful.

Record *14* of figure 5 that was obtained at a low sweep speed (cf. record *20*, below) presents the Et spike with two phases. The second positive phase was certainly not referable to a change in the potential of the second recording electrode (r_2); the tetraethyl-ammonium solution was not placed in contact with the last 10 mm of nerve and for this reason conduction of impulses in the end segment of the nerve did not take place (cf. also fig. 6). In view of this fact one might conclude that the second phase was referable to a change in the membrane potential at the level of the first recording electrode (r_1), were it not that longitudinal polarization of the core in the segment $r_1r'_1$ and in the next 1 or 2 mm of nerve could also have been the mechanism of production of the second phase (cf. '47, section V.6). The need of taking into account the possible rôle of core polarization arises not only from the fact that core polarization plays an important rôle in the case of A fibers, but also from the fact that the second phase of the spike of record *14* was produced during the time of activity of the last few millimeters of restored nerve.

Record *16* presents the spike recorded with the oscillograph connected to points r'_1 and r_2; as can readily be noted the spike in record *16* corresponds to the positive phase of the spike in record *14*; on the other hand, records *15* and *17*, that were obtained with the oscillograph connected to electrodes r_1 and r'_1 present diphasic spikes in which the second phase is more pronounced but has approximately the same temporal course as the second phase of the spike in record *14*. These facts might have been referable to a coincidence, in the sense that if the $r_1r'_1$ distance had been longer the second phase of record *14* would not have appeared at the same time as the spike of record *16*, but at any rate it is clear that the detailed analysis of the Et spike cannot lead to conclusive results until experiments especially designed for this purpose have been carried out.

In view of these observations, it is clear that the comparison of the durations of spikes recorded under different experimental conditions cannot be made with any degree of accuracy. Fortunately, the difference between the duration of the Et spike in nerve restored by tetraethyl-ammonium and in nerve restored by sodium is so great that inspection of the records is sufficient to warrant the conclusion that in nerve restored by tetraethyl-ammonium both the ascending and the descending leg of the spike have much greater duration than in normal nerve or in nerve restored by sodium. The results to be presented in section 10,b will remove any possible doubt on the validity of the conclusion.

The fact that tetraethyl-ammonium ions lengthen both legs of the spike is important. According to the results of the analysis presented elsewhere ('47, section XIV.6), the duration of the interval of time between the inflection point of the spike and the crest of the spike may be regarded as the duration of the alteration (nerve impulse); therefore, the alteration has a greater duration in nerve restored by tetraethyl-ammonium than in normal nerve. On the other hand, the increased duration of the descending leg of the spike indicates that in nerve restored by tetraethyl-ammonium the recovery of the membrane potential takes place at a much lower rate than in nerve restored by sodium.

The third difference between nerve restored by tetraethyl-ammonium ions and nerve restored by sodium ions is that nerve restored by tetraethyl-ammonium is very susceptible to fatigue. Repetitive stimulation at 10-second intervals is usually sufficient to produce a marked decrease in the spike height and in the speed of conduction; moreover, very few of the restored fibers are able to follow stimulation at 5-second intervals, which indicates that the absolutely refractory period of many of the restored fibers is longer than 5 seconds. Fibers of slow conduction restored by sodium usually are able to follow stimulation at 2-second intervals without readily detectable signs of fatigue (cf. below, section 10).

b. Restoration by ions having two or more ethyl groups. None of the quaternary ammonium ions having only one ethyl group, that have been used in the research presented here,[5] is able to restore the excitability of nerve fibers that have become inexcitable in a sodium-free medium. In order to obtain restoration it is necessary to use ions having two or more ethyl groups. The effectiveness of the ions increase with the number of ethyl groups that they contain.

In the experiment illustrated by figures 6 to 8 a comparison was made of the effects of ions IV and V and of sodium upon a nerve that had become inexcitable in a 0.11 M solution of ion II. Figure 6 illustrates stages of the restoration of the excitability of the nerve fibers. The observations were begun after the nerve had been deprived of sodium for 17 hours.

[5] Quaternary ammonium ions having only one ethyl group have been prepared by ethylation of pyridine, niacinamide and coramine. None of these ions is a restoring ion (cf. Part II).

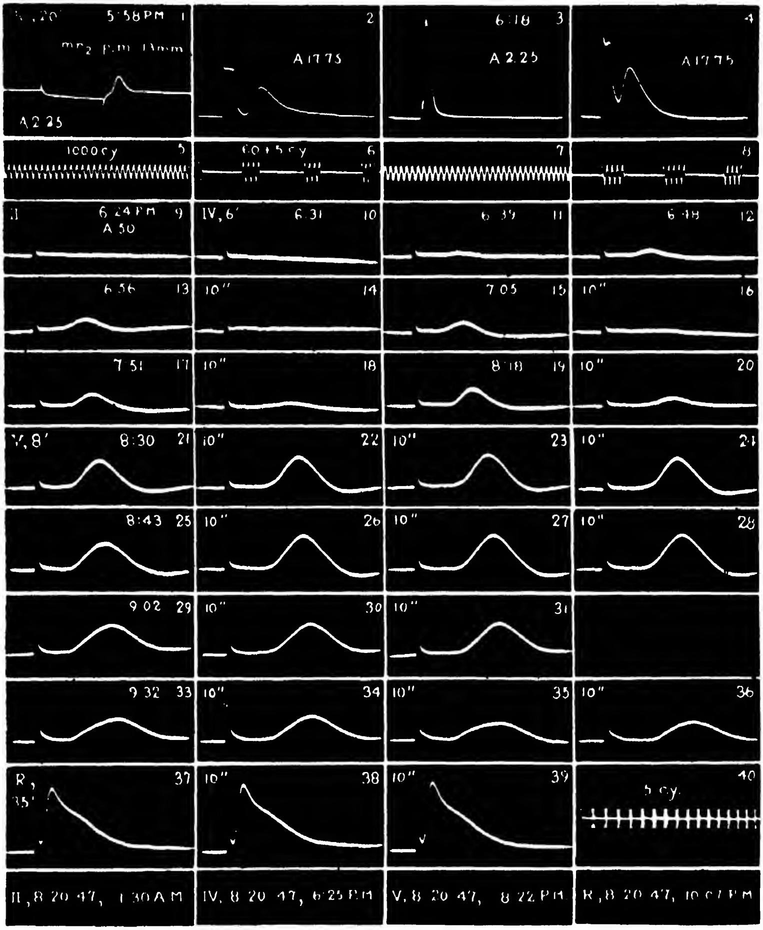


FIG. 6. Restoration of the excitability of nerve fibers deprived of sodium in a 0.11 M solution of ethyl-trimethyl-ammonium (fig. 1, II).

1 to *4*, restoration of the central segment; record *1* was obtained at 5:58 P.M., 20 minutes after the segment had been placed in contact with Ringer's solution. *1*, response of fibers of fast conduction to the break of the anodal current; *3*, spike of fibers of fast conduction initiated by a brief pulse of cathodal current; *2* and *4* spikes of fibers of slow conduction initiated by rectangular pulses of cathodal current; the initial deflection was produced by the electrotonic potential of fibers of the A group.

9, lack of conducted response in the peripheral segment before the application of the restoring solution; *10* to *20*, restoration by methyl-triethyl-ammonium; *21* to *36*, enhancement of the restoration by tetraethyl-ammonium; *37* to *39*, enhancement of the restoration by Ringer's solution.

The restoring solutions were not applied to the last 6 mm of the r_1r_2 segment.

The central segment of the nerve was placed in contact with Ringer's solution at 5:38 P.M. and records *1* to *4* were obtained with the first recording electrode at point m (fig. 3,II), at the indicated times. At the time when records *1* and *2* were obtained the recovery of the A fibers still was in an early stage; the fibers were inexcitable to cathodal currents, but a number of fibers produced impulses in response to the opening of anodal current currents of the appropriate magnitude and duration (cf. below, section 9,d); the recovery of the fibers of slow conduction, however, was quite advanced (record *2*). After 30 additional minutes of the action of sodium the recovery of both classes of fibers became practically complete (records *3*, *4*).

The peripheral segment of the nerve was placed in contact with a 0.11 M solution of ion IV (methyl-triethyl-ammonium) at 6:25 P.M., immediately after record *9* had been obtained. No conducted spike appears in record *10*, but a definite response can be seen in record *11*. The spike was observed to increase progressively in size (records *12*, *13*, *15*, *17*, *19*) although at a relatively low rate. On the other hand, the restored fibers were exceedingly susceptible to fatigue; the two records of each one of the 4 pairs *13*, *14*; *15*, *16*; *17*, *18*, and *19*, *20* were obtained 10 seconds apart; in each case the second record presents a spike much smaller than that in the first, indicating that the absolutely refractory period of the majority of the restored fibers was longer than 10 seconds.

The peripheral segment of the nerve was placed in contact with a 0.11 M solution of ion V (tetraethyl-ammonium) at 8:22 P.M. A comparison of records *19* and *21* shows that after 8 minutes the action of tetraethyl-ammonium had been able to increase considerably the number of responding fibers. Furthermore, tetraethyl-ammonium had been able to decrease the fatigability of the nerve fibers; records *21* to *24* were obtained at 10-second intervals, and instead of the decrease in the height of the spike previously caused by repetitive stimulation (records *19*, *20*), a slight increase was observed. Since the increase in the spike height was accompanied by a decrease in the speed of conduction, a possible interpretation of the phenomenon, based on analogy with observations made with A fibers ('47, sections XIV.6, XV.8), would be this. The decrease in the speed of conduction was the sign of mild cathodal depression (relative refractoriness), which caused an increase in the stimulation threshold but was not sufficient to decrease the number of conducting fibers; the increase in the spike height indicated that a larger amount of L fraction collapsed during the second and following spikes than during the first spike of the train. The restoring action of tetraethyl-ammonium was nearly maximal at the time when records *21* to *24* were obtained; it is true that the response still increased slightly in the following 13 minutes (records *25* to *28*); but later both the spike height and the speed of conduction decreased progressively (records *29* to *36*). This phenomenon was probably referable to a continued increase in the L fraction (cf. sections 6,b and 10).

The peripheral segment of the nerve was placed in contact with Ringer's solution at 10:07 P.M. and records *37* to *39* were obtained 35 minutes later. No restoration of excitability of A fibers had taken place yet, but the state of

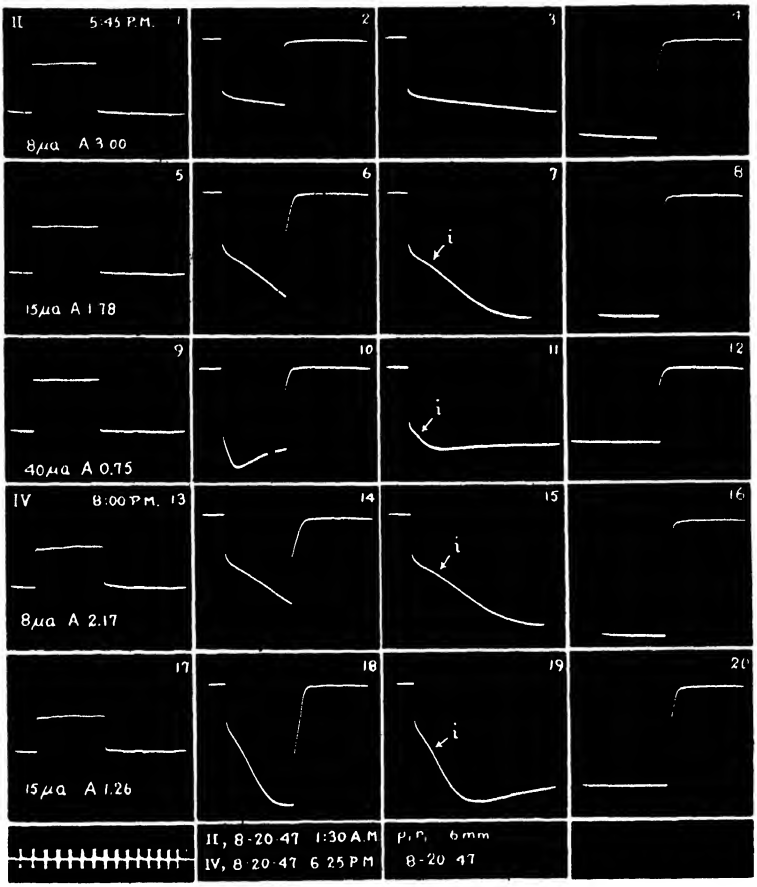


FIG. 7. Electrotonic potentials in a nerve kept in 0.11 M ethyl-trimethyl-ammonium chloride, before (*1* to *12*) and after restoration by methyl-triethyl-ammonium chloride (*13* to *20*). The arrows labelled *i* indicate the inflection points in the tracing of the anelectrotonus which resulted from restoration of the polarizability of the membrane by the applied current. Note the Et spikes superposed upon the catelectrotonus in records *13* and *17* (cf. fig. 24, *9*, Et).

the fibers of class Et had undergone a marked improvement, that revealed itself chiefly in the spectacular increase in the speed of conduction of impulses. The spike height also increased, probably because an additional number of Et

fibers became able to conduct impulses after Na^+ ions were made available to them.

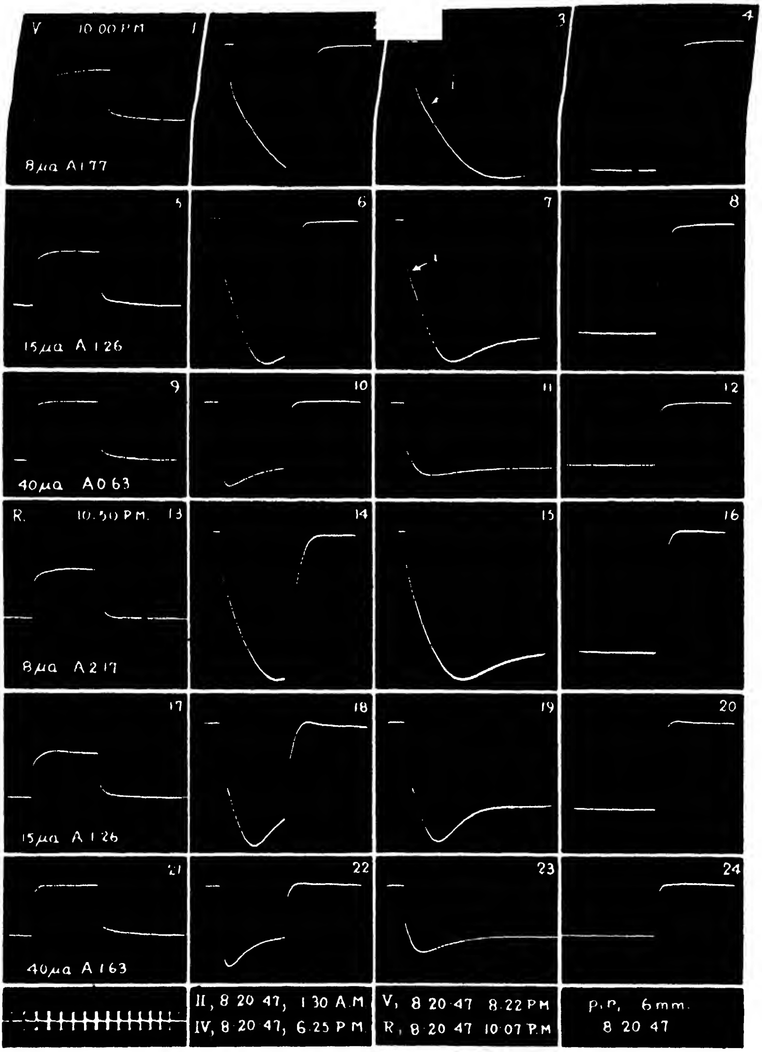


Fig. 8. (Continuation of fig. 7.) Electrotonic potentials after restoration by tetraethyl-ammonium chloride (*1* to *12*) and by Ringer's solution (*13* to *24*).

Figures 7 and 8 illustrate the progressive changes in the electrotonic potentials that were observed during the experiment. It will be convenient to consider now only the records obtained with 8 and 15 μa currents (cf. below, sec-

tion 9,c). Records *1* to *8*, figure 7, which were obtained with the nerve fibers still surrounded by the solution of ethyl-trimethyl-ammonium chloride, are quite similar to records *1* to *8* of figure 4; the only difference was that in the case of the nerve used to obtain the records of figure 7 the polarizability of the membrane was slightly greater; hence the greater height of the slow anelectrotonus in the records of figure 7. This difference in the behavior of the two nerves is doubtless referable to the fact that one nerve had been kept in the sodium-free medium for 29 hours (fig. 4) and the other for 16 hours (fig. 7). The restoring action of the 0.11 M solutions of methyl-triethyl-ammonium (fig. 7, *13* to *20*), tetraethyl-ammonium (fig. 8, *1* to *12*) and sodium ions (fig. 8, *13* to *24*) were placed in evidence by the increase in the L fraction of the membrane potential, or otherwise stated, in the height of the slow catelectrotonus (cf. fig. 7, *1*, *5*; *13*, *17*; fig. 8, *1*, *5*; *13*, *17*) and by the increase in the polarizability of the membrane, which resulted in an increase in the rate of establishment and in the magnitude of the slow anelectrotonus. If figures 7 and 8 are examined in some detail, it will be observed that after every change in solution the smaller of the two currents (8 μa) produced slow electrotonus having the same temporal course as that which had been produced previously by the larger current (15 μa). The records to be compared are: *6* to *8* with *14* to *16* in figure 7; *18* to *20* of figure 7 with *2* to *4* of figure 8 and *6* to *8* with *14* to *16* in figure 8. This phenomenon is an example of a general rule according to which the polarizability of the boundary at which the slow anelectrotonus is established by the applied current increases with the value of the L fraction of the membrane potential. The progressive sharpening of the maximum of the anelectrotonus indicated that the intensity of the E_3 reaction also increased with increasing value of the L fraction (cf. below, section 9,c and d).

All the quaternary ammonium ions with two ethyl groups listed in figure 1 have proved to be able to effect a partial restoration of nerve deprived of sodium. If the nerve is then submitted to the action of ions containing three or 4 ethyl groups the state of the nerve fibers undergoes a further improvement.

In the experiment illustrated by figures 9 and 10, after the nerve had been kept in the 0.11 M solution of ethyl-trimethyl-ammonium for 18 hours, the polarizability of the membrane had become so low that the applied currents produced only very little slow anelectrotonus (fig. 9, *2* to *4*; *6* to *8*); moreover, the fact that the slow anelectrotonus increased continuously during the flow of the applied current (fig. 9, *3*, *4*; *7*, *8*) indicated that the intensity of the E_3 reaction was very small. The action of dimethyl-diethyl-ammonium ions (fig. 1, III) resulted in a small but clearly noticeable improvement of the state of the nerve fibers; it is true that the catelectrotonus (fig. 9, *9*, *13*) did not acquire a slow component, but the height of the slow anelectrotonus (records *10* to *12* and *14* to *16*) underwent a significant increase. After the action of tetraethyl-ammonium ions upon the nerve had lasted for 100 minutes the increase in the L fraction of the membrane potential became sufficient for the appearance of

slow components in the catelectrotonus (records *17*, *21*). The anelectrotonus produced by the 15 μa current passed through a definite maximum during the

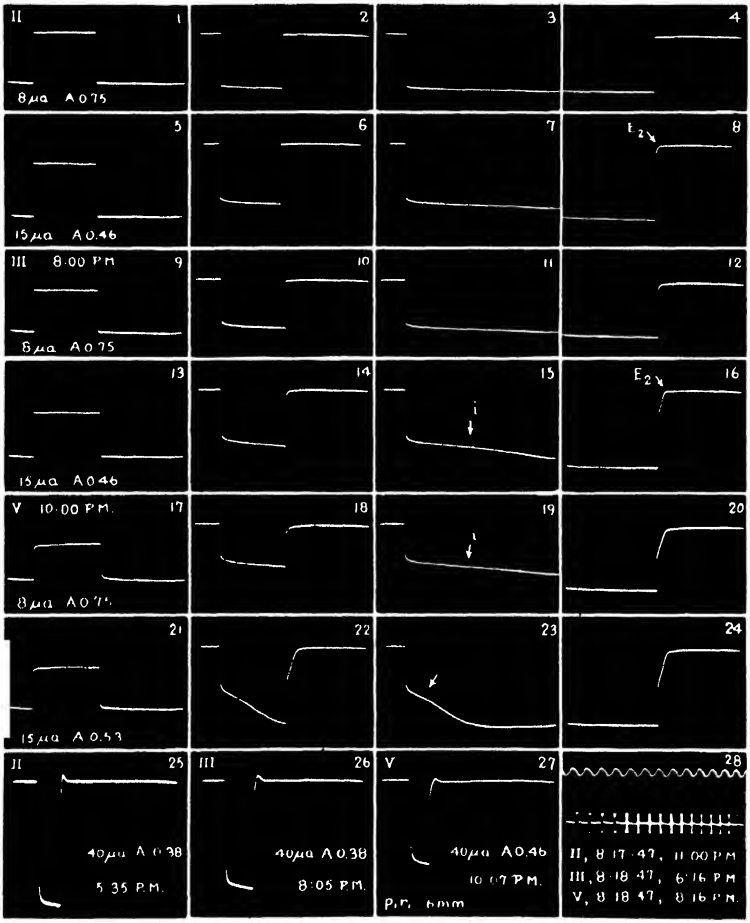


FIG. 9. Electrotonic potentials in a nerve kept in a 0.11 M solution of ethyl-tri-methyl-ammonium chloride, before (*1* to *8*) and after restoration by dimethyl-diethyl-ammonium chloride (*9* to *16*) and by tetraethyl-ammonium chloride (*17* to *24*). *25* to *28*, initiation of impulses in A fibers by the break of the anodal current. The upper time line of record *28* (200 cycles per second) applies to records *25* to *27*, the lower time line (5 cycles), to records *1* to *24*.

flow of the applied current (records *23*, *24*), indicating that the nerve fibers had become able to produce an E_3 reaction of considerable intensity.

No nerve fiber was able to conduct impulses into the peripheral segment of

the nerve at the time when record *1* of figure 10 was obtained; record *2*, however, shows that a few Et fibers had become able to conduct impulses 6 minutes after dimethyl-diethyl-ammonium ions had been available to them. The number of restored fibers increased progressively during the following 30 minutes (records *3* to *5*) to remain unchanged during the following 70 minutes (records *6* to *8*). The nerve was then placed in contact with 0.11 M tetraethyl-ammonium chloride, which resulted in a rapid increase in the number of responding fibers (records *9* to *12*). The height of the conducted spike remained nearly constant for some time (records *13*, *14*); later, it decreased slightly. A compari-

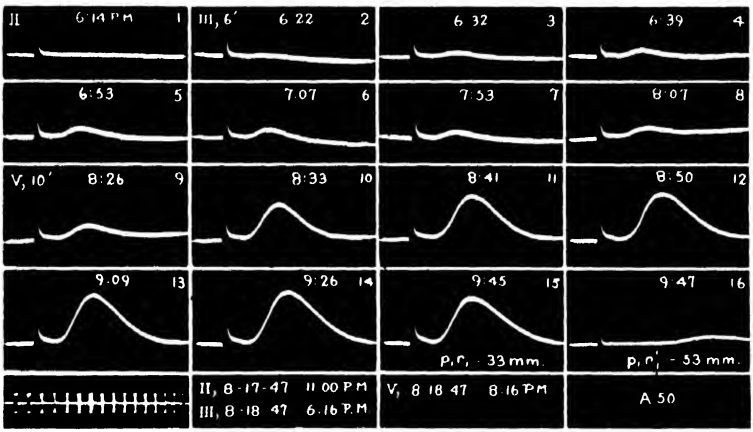


FIG. 10. Restoration of the ability to conduct impulses to fibers of class Et, rendered inexcitable in a solution of ethyl-trimethyl-ammonium ions (record *1*) by dimethyl-diethyl-ammonium ions (records *2* to *8*) and by tetraethyl-ammonium ions (records *9* to *16*). The conduction distance for records *1* to *15* was 33 mm, that for record *16*, 53 mm. The amplification was constant (A50).

son of the shock spike times in records *15* and *16*, that were obtained with conduction distances of 33 and 53 mm, respectively, shows that the maximal speed of conduction of the restored fibers was 25 mm per second.

In the experiment illustrated by figure 11 the nerve was rendered inexcitable by its being kept in 0.11 M ethyl-trimethyl-ammonium chloride for 14 hours; the restoration was effected by means of methyl-ethanol-diethyl-ammonium ions (fig. 1, VIII), and was enhanced by making ethanol-triethyl-ammonium ions (fig. 1, IX) available to the nerve.

Records *1* to *4* of figure 11 present spikes of fibers of fast conduction (*1*, *3*) and of fibers of slow conduction (*2*, *4*), recorded with the first recording electrode at a point m (fig. 3, II), at two stages of the restoration of the central segment of the nerve by Na^+ ions. As is routinely observed in experiments of this kind

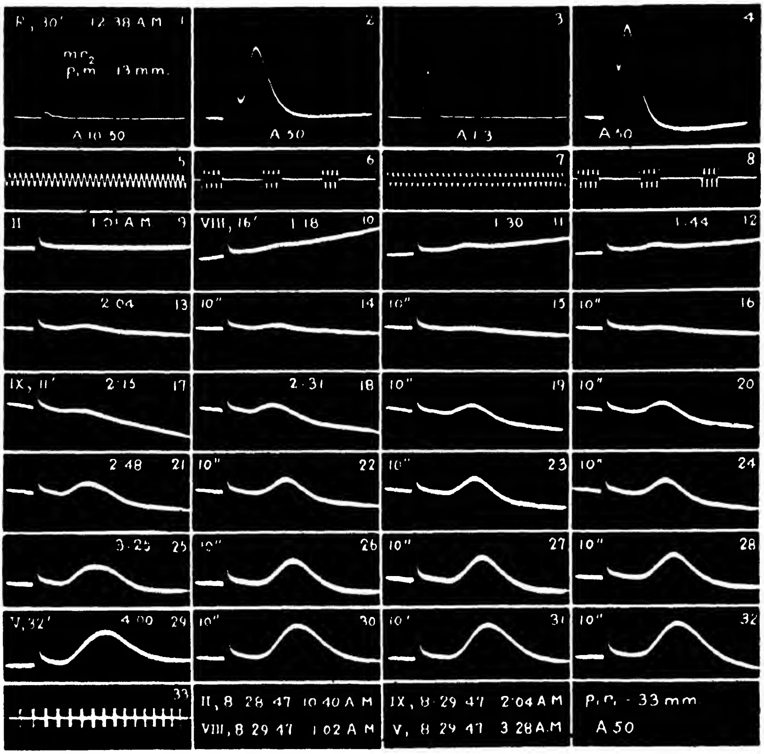


FIG. 11. Restoration of the excitability of a nerve rendered inexcitable in ethyl-trimethyl-ammonium chloride.

1 to *4*, restoration of the excitability of the central segment by Ringer's solution. Records *1* and *2* were obtained at 12:38 A.M , 30 minutes after the solution was placed in contact with the nerve and records *3* and *4* at 12:54 A.M. *1* and *3*, responses of fibers of fast conduction; *2* and *4*, responses of fibers of slow conduction. Note that the recovery of the fibers of slow conduction was more rapid than that of fibers of fast conduction. In all probability, the positive phase of the spike in record *4* was referable to a temporary hyperpolarization of the membrane (positive after-potential).

9, absence of conducted response in the peripheral segment of the nerve; *10* to *16*, restoration of Et fibers by methyl-ethanol-diethyl-ammonium ions; *17* to *28*, enhancement of the restoration by ethanol-triethyl-ammonium ions; *29* to *32*, enhancement of the restoration by tetraethyl-ammonium ions. The conduction distance (33 mm), the amplification (A50) and the sweep speed (record *33*, 5 cy) were constant for records *9* to *32*.

the recovery of the fibers of slow conduction progressed more rapidly than that of the fibers of fast conduction.

Record *9* places in evidence the inability of the nerve fibers to conduct im-

pulses into the peripheral segment of the nerve. A few fibers became able to conduct impulses 16 minutes after the nerve had been placed in contact with the solution of ion VIII; the number of restored fibers increased somewhat during the following 25 minutes (records *11*, *12*); thereafter it remained stationary (cf. record *13*). The great fatigability of the restored fibers resulted in a marked decrease of the spike height during repetitive stimulation at 10-second intervals (records *14* to *16*). Corresponding to the small number of fibers of class Et that were restored by ion VIII, the electrotonic potentials of the fibers of group A did not show any readily detectable improvement.

Ion IX, that contains three ethyl groups, produced a marked increase in the number of responding fibers (records *17*, *18* and *21*); it also reduced the fatigability of the nerve fibers, since no decrease in the spike height is noticeable in the records of the series *18* to *20*, *21* to *24*, and *25* to *28* that were obtained at 10-second intervals. A further increase of the conducted response was produced by tetraethyl-ammonium (records *29* to *32*).

Finally, in the experiment illustrated by figure 12 the restoration was initiated by the use of a 0.11 M solution of ion XII (diethyl-diethanol-ammonium) and was enhanced by the use of ion IX (ethanol-triethyl-ammonium). Records *1* and *3* show that the restoration of the central segment was practically complete 40 minutes after Na^+ ions had been made available to it. The restoring action of ion XII upon the peripheral segment developed at a relatively low rate since a readily detectable, conducted spike was not observed until the ion had acted for 18 minutes (record *8*); moreover, the number of conducting fibers increased little during the following 55 minutes (records *9*, *11*, *13*), and the fatigability of the restored fibers was great. Ion IX produced a marked increase in the number of responding fibers; in addition, it prevented the decrease in the spike height during repetitive stimulation at 10-second intervals (records *15* to *27*).

c. Comment. If no other information were available, the experimental facts presented in this section could be interpreted as a proof that, when it forms part of a quaternary ammonium ion, the ethyl group plays a specific rôle in nerve physiology. Additional experiments, however, have shown that restoration of the excitability of Et fibers of nerves deprived of sodium can also be effected by two quaternary ammonium ions that do not contain ethyl groups, tetrapropyl-ammonium and tetrabutyl-ammonium (cf. section 7). Thus, the ethyl group cannot be said to play a specific rôle; the only general conclusion that can be drawn from the experimental results is that introduction of ethyl groups in quaternary ammonium ions as well as the introduction of other groups to be mentioned in sections 6 and 7 modifies the properties of the ions in such a manner that they become able to substitute for sodium in certain aspects of nerve function.

In which manner the replacement of methyl by ethyl groups changes the properties of the ions listed in row 1 of figure 1 is a problem that only experts

in the theory of organic chemistry may attempt to solve. The changes in properties are more profound than is indicated by the facts heretofore reported. It has been shown than the ability of ions III, IV and V to restore the excitability of Et fibers deprived of sodium increases with the number of ethyl groups

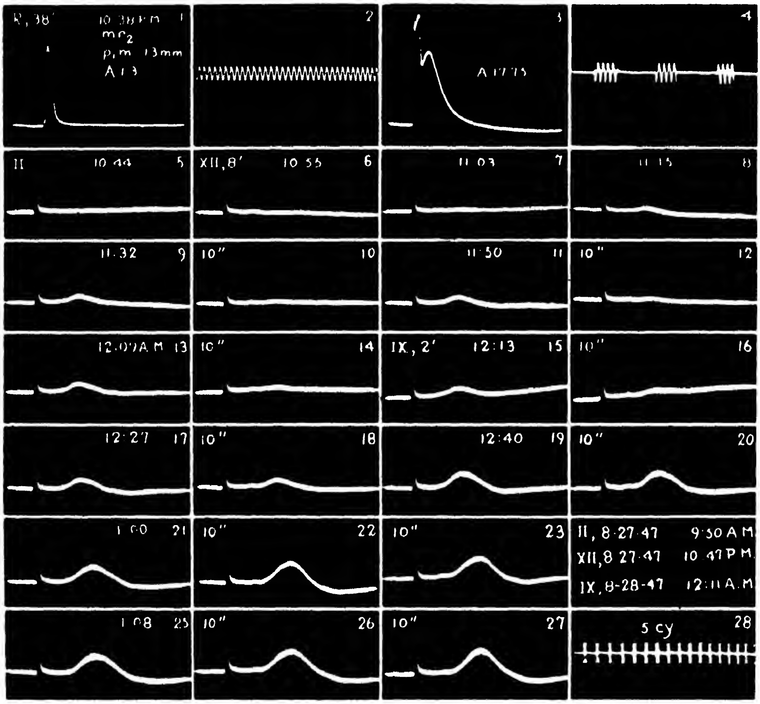


FIG. 12. Restoration of the excitability of a nerve rendered inexcitable in 0.11 M ethyl-trimethyl-ammonium chloride.

1 and *3*, responses recorded at point m, 38 minutes after the central segment had been placed in contact with Ringer's solution; time line *2* (1000 cy) applies to record *1*; time line *4* (60 + 5 cy) to record *3*.

5, absence of conducted response in the peripheral segment of the nerve; *6* to *14*, restoration by diethanol-diethyl-ammonium; *15* to *27*, enhancement of the restoration by ethanol-triethyl-ammonium. The amplification (A50) and the sweep speed (record *28*) were constant for records *5* to *27*.

in the ion; also the ability of the ions to improve the state of A fibers deprived of sodium increases with the number of ethyl groups. In these respects ions III, IV and V may be said to belong to a continuous series. A different situation prevails if the long lasting effects of the three ions are compared.

Nerves left in a 0.11 M solution of ion III become inexcitable much in the

manner of nerves left in solutions of ions I or II, in spite of the fact that ion III is able partially to restore nerves that have become inexcitable in solutions of ions I or II. As will be shown in section 5,c, the excitability of nerve fibers that have become inexcitable in a solution of ion III can be restored by tetraethyl-ammonium (Et fibers) as well as by sodium (all fibers). In nerves kept in a 0.11 M solution of tetraethyl-ammonium chloride only the A fibers lose their ability to conduct impulses; the fibers of class Et remain excitable for many hours after the effect of the lack of Na^+ ions should have rendered them inexcitable. If Na^+ ions are made available to the nerve, the A fibers regain their ability to conduct impulses (cf. section 10).

In sharp contrast with these results all the fibers of nerve kept in a 0.11 M solution of ion IV for longer than 14–16 hours lose their excitability irreversibly, since neither tetraethyl-ammonium nor sodium is able to restore the excitability, even after either has been allowed to act for several hours. In point of fact, the deleterious effect of long lasting action of ion IV goes so far as to cause the loss of the core conductor properties of the nerve fibers, i.e., to produce a far reaching disintegration of the membrane. This phenomenon will be analyzed in detail in section 4,d in reference to ion VIII, that has a deleterious action similar to that of ion IV. It will be shown in section 8,b that ion IV is a strong depolarizing agent.

Even though a detailed explanation of the discontinuity in the changes of properties of ions III, IV and V cannot be offered at present, there can be no doubt that the existence of the discontinuity is an important fact, particularly because a similar discontinuity exists in the series VI to IX and X to XIII (cf. sections 4 and 5).

4. *Quaternary Ammonium Ions of the Choline Group*

This section deals with experiments done with the use of the ions listed in row 2 of figure 1 and of ions XXIV and XXV (fig. 2). All these ions have an ethanol group, that is combined with an acetyl group in the case of ions XXIV and XXV; they differ in the number of methyl and ethyl groups that they contain.

a. An observation on "isotonic" solutions. Repeatedly, the present writer ('44, '47) has used solutions of choline chloride as "indifferent," sodium-free solutions in order to analyze the effects of the lack of sodium ions. The concentration of choline chloride used in most cases, 2.5% (0.18 M), is rather high, since the concentration of Ringer's solution is only 0.11 M. The choice of the concentration of choline chloride was based upon the results of a series of experiments in which nerves, that had been rendered inexcitable by strongly hypotonic solutions, were restored by transfer to solutions of choline chloride. The best results were obtained with 2.5% choline chloride and since this solution proved to be an adequate, sodium-free medium, it has been routinely used for several years. In the research presented here, in order to keep constant the molar concentra-

tions of all the quaternary ammonium salts used, choline chloride has been used at the 0.11 M concentration (1.6%). Thus, the opportunity was given to compare in detail the effects upon nerve of choline chloride at two different concentrations, 0.11 M and 0.18 M.

No readily detectable difference has been observed between the properties of nerves kept in one and in the other solution; indeed, no significant difference was observed even when the two nerves were taken from the same frog and were examined simultaneously. The tests done consisted only in oscillographic analysis of the properties of the nerves and in measurements of changes in the membrane potential; therefore, the possibility exists that tests done with the use of chemical methods might reveal differences, for example in the water content, but whatever the differences might be, they certainly are of little significance for nerve function.

Since the osmotic pressures of 0.11 and 0.18 M solutions are widely different, the fact that nerve fibers do not seem to "detect" the difference between the two solutions is remarkable; after some thought, however, one cannot fail to realize that this fact is not more remarkable than another known fact. Nerves kept in a strongly hypotonic solution, practically in distilled water, do not deteriorate irreversibly unless they are kept in the solution for several hours, and even after all the nerve fibers have lost their ability to conduct impulses, complete recovery can be effected by transferring the nerve to Ringer's solution ('47, section IV.1). The immediate conclusion to be drawn from the two facts is that the osmotic equilibrium of the nerve fibers has peculiarities of its own; it should not be directly compared with the osmotic equilibrium in other biological systems, such as the red blood corpuscles or those plant cells which are ordinarily used in osmotic studies.

It will be useful to add a few remarks. The nerve fibers may maintain their anatomical integrity and functional ability for many hours in solutions of concentrations as widely different as 0.11 M and 0.18 M, which indicates that the nerve fibers are quite insensitive to changes in the osmotic pressure of their external medium. On the other hand, all the nerve fibers may remain excitable for a number of hours in solutions of saccharose or of choline chloride and the Et fibers for a much longer time in solutions of tetraethyl-ammonium chloride, i.e., in solutions that do not contain any of the solutes known to be normally present in blood serum. Only one explanation of these facts is possible (cf. '47, section I.14 and Concluding Notes). To a large extent the nerve fibers are self-contained mechanisms that maintain their membrane potential and their functional ability by means of their own metabolism. Blood-perfused nerve fibers probably obtain their metabolic substrates continuously from their external medium; excised nerve fibers kept in suitable artificial media can use only their internal stores of metabolic substrates, but these stores are sufficient to maintain the metabolism in approximately normal fashion for many hours, since the

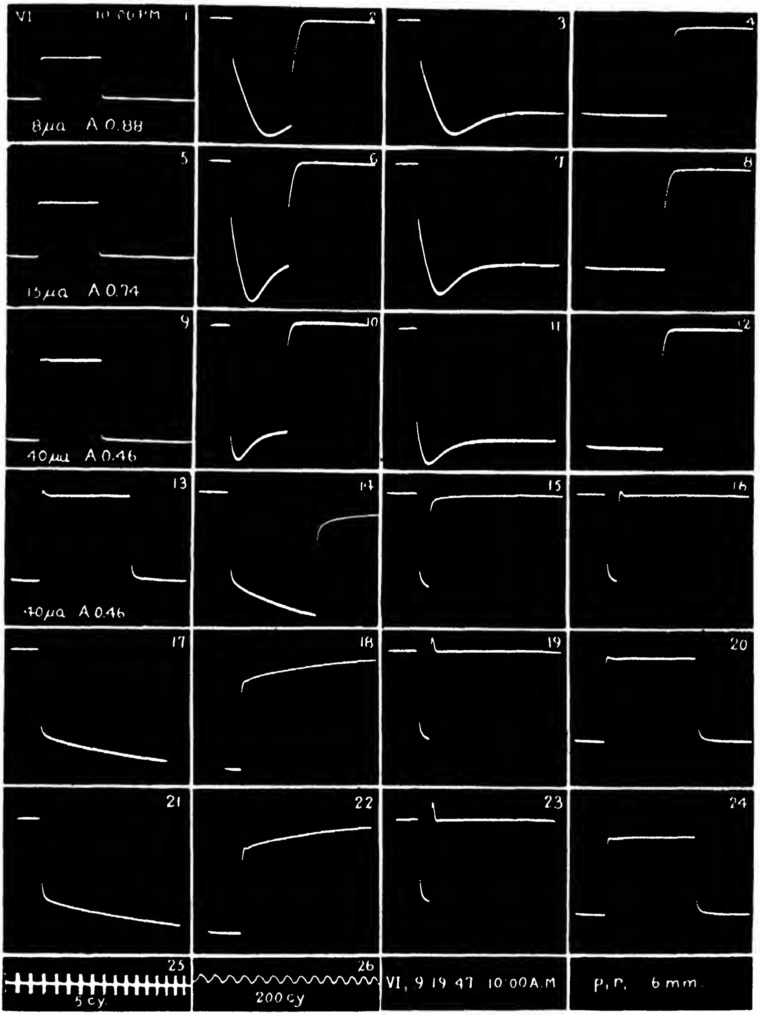


FIG. 13. Electrotonic potentials in a nerve kept in 0.11 M choline chloride for 12 hours (*1* to *12*) and initiation of impulses by the closure of the cathodal current (*13*, *20*, *24*) and the opening of the anodal current (*14* to *23*); note that the impulses failed to propagate themselves along the nerve, obviously because the action current was insufficient to reach the threshold of the nerve fibers; further explanation in text. Time line *25* applies to records *1* to *12*; time line *26*, to records *13* to *24*.

nerve fibers maintain their anatomical and functional integrity provided only that oxygen be available to them. Under conditions such as these it is logical to assume that, except in the case of substances with specific action, changes in

the external medium of the nerve fibers result only in unimportant internal changes. In the particular case of water, the experimental evidence indicates that small changes in the external concentration of water do not result in significant internal changes, or that if they do, the internal changes are compensated by the activity of the metabolic mechanisms, much in the manner that the exalted activity of the metabolic mechanisms can compensate for the effects of relatively large increases in the external concentration of potassium (cf. '47, section I.9 and I.15).

b. Effects of replacing the methyl groups of choline by ethyl groups. Nerves kept in sodium-free solutions of choline become reversibly inexcitable while they keep their membrane potential at the normal level. In this respect choline acts like ions I and II; there is, however, a slight difference between the effects of choline and the effects of the inert ions (I, II and X), which deserves mention. In the case of the A fibers the difference is revealed by the behavior of the electrotonic potential; in the case of the fibers of slow conduction the difference consists (1) in that the fibers lose their ability to conduct impulses later in a solution of choline than in a solution of ions I, II or X, and (2) in that if the nerves are kept in choline chloride for a few hours after all the fibers have become inexcitable, the restoration of excitability of Et fibers by quaternary ammonium ions is only partial.

Records *1* to *12* of figure 13 and *1* to *8* of figure 14 present the electrotonic potentials of nerves kept in a 0.11 M solution of choline chloride for 12 and 16 hours respectively. The cathodal records (*1*, *5*, *9*) of figure 13 are similar to the cathodal records of, for example figure 4 (*1*, *5*); the anodal records in the two figures are different. In the case of records *2* to *4*, *6* to *8* and *10* to *12* of figure 13 the slow anelectrotonus was established at a high rate and passed through a maximum even in the case of the 8 μa current. Thus, the polarizability of the membrane and the effectiveness of the nerve reaction are greater in a nerve kept in choline chloride than in a nerve kept in tetramethyl-ammonium chloride. The difference, however, is not great. The lack of overshootings after the end of the polarization (fig. 13, *2*, *4*; *6*, *8*; *10*, *12*) proves that also in the choline-treated nerve the nerve reaction was established in a defective manner; on the other hand, when the polarizability of the membrane was tested with the 1 μa current it proved to be quite low, since this current produced only a very small slow anelectrotonus, that increased continuously during the flow of the current (cf. '47, fig. VIII.13).

The polarizability of the membrane of nerves kept in choline chloride decreases progressively with advancing time, as is shown by the fact that the height of the slow anelectrotonus undergoes a progressive decrease; however, the temporal course of the anelectrotonus produced by relatively large currents (8, 15 μa) remains essentially unchanged. Records *2* to *4* and *6* to *8* of figure 14 show that after the nerve had been in 0.11 M choline chloride for 16 hours the slow anelectrotonus had a small height, but its temporal course was essen-

tially equal to the course observed in earlier stages, since with the two currents that were used the anelectrotonus displayed a maximum. In this respect the state of nerves kept in choline chloride is different from that of nerves kept in

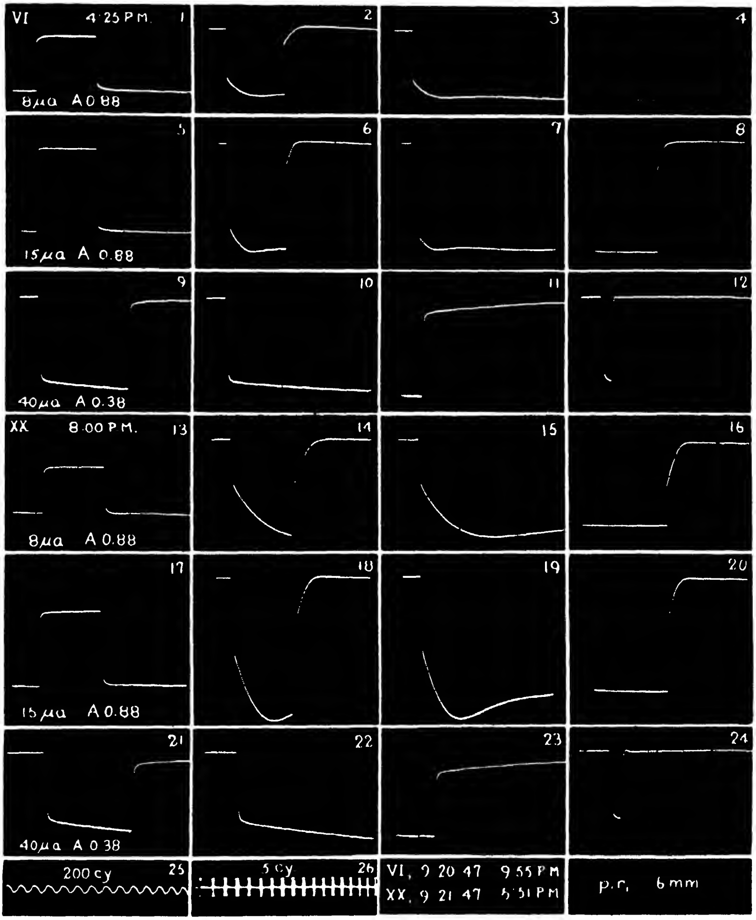


FIG. 14. Electrotonic potentials in a nerve kept in 0.11 M choline chloride for 16½ hours, before (*1* to *12*) and after restoration by phenyl-triethyl-ammonium ions. Time line *25* applies to records *9* to *12* and *21* to *24*; time line *26* to records *1* to *8* and *13* to *20*.

tetramethyl-ammonium chloride (fig. 4, *2* to *4*, *6* to *8*). At the present state of knowledge the exact significance of the difference cannot be understood; it is important, however, to know that the difference exists.

The quaternary ammonium ions of the restoring type produce with choline-

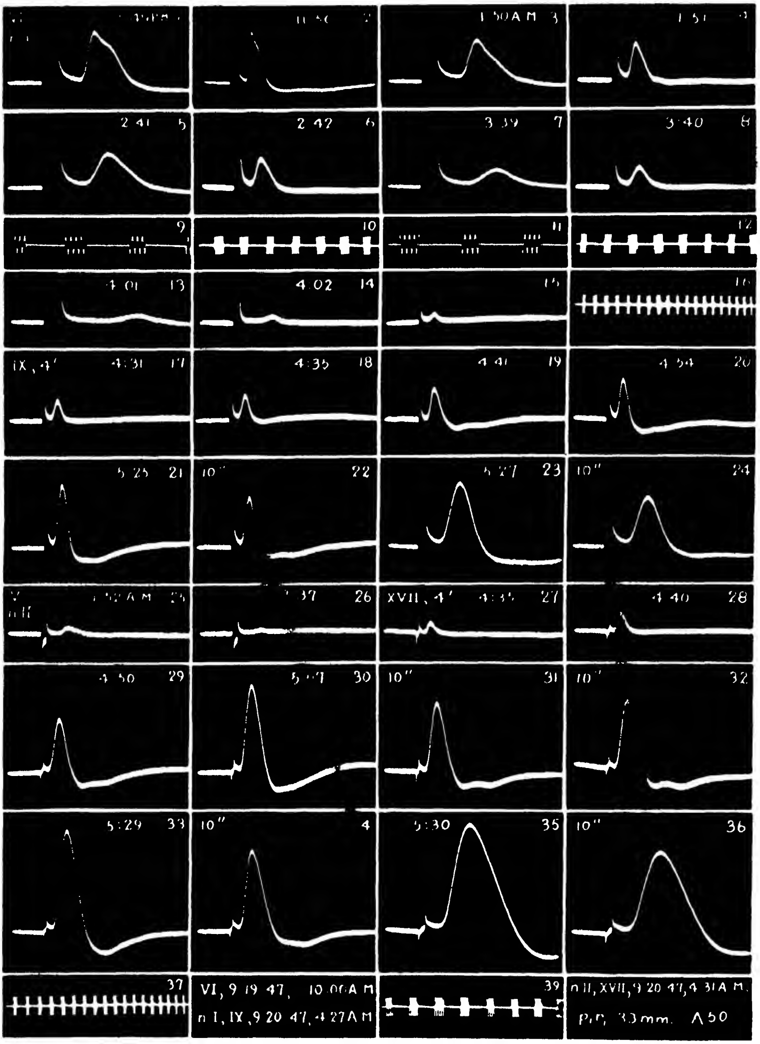


FIG. 15. Observations made in the experiment of figure 13. *1* to *15*, terminal phases of the development of inexcitability of fibers of slow conduction in nerve I (n.I). Time lines *9* and *11* correspond to records *1*, *3*, *5*, *7* and *13*; time lines *10*, *12* to records *2*, *4*, *6*, *8* and *14*; time line *16* to record *15*. *17* to *24*, restoration of nerve I by ethanol-triethyl-ammonium ions. Records *17* to *22* were obtained with the sweep speed record *16*, and records *23*, *24* with the speed of record *12*.

25, *26*, terminal phases of the development of inexcitability of the fibers of slow conduction of nerve II (n.II); *27* to *36*, restoration of nerve II by n-propyl-triethyl-ammonium ions. Records *25* to *34* were obtained with the speed of record *37*; records *35*, *36*, with that of record *39*. The conduction distance (33 mm) and the amplification (A50) were constant. The Roman numeral on the upper left corner of record *25* should be VI.

treated nerves essentially the same results as with nerves kept in solutions of ions I or II. Records *13* to *20* of figure 14 show that after the nerve had been in contact with a 0.11 M solution of phenyl-triethyl-ammonium chloride for two hours the electrotonic potentials had made a marked change toward normality.

In solutions of choline chloride the nerve fibers lose their excitability in the same order as in other sodium-free solutions, a 0.22 M solution of saccharose or 0.11 M solutions of compounds I, II, III, VII, X or XIII to XV. The fibers of the A group are the first to lose their ability to conduct impulses; a few hours later also the fibers of slow conduction become inexcitable (cf. end of section 2). A remarkable fact is that long after the A fibers have lost their

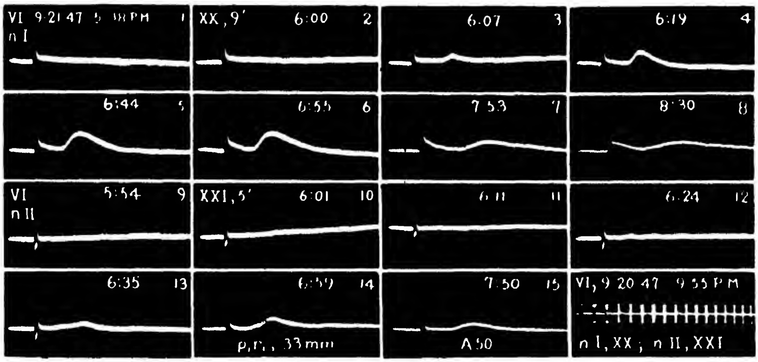


FIG. 16. Observations made in the experiment of figure 14. *1*, absence of conducted response in the peripheral segment of nerve I; *2* to *8*, restoration by phenyl-triethyl-ammonium ions, that were made available to the nerve at 5:51 P.M. *9*, absence of response in the peripheral segment of nerve II; *10* to *15*, restoration by β-phenylethyl-triethyl-ammonium ions, that were made available to the nerve at 5:56 P.M.

ability to conduct impulses a small group of them can still be stimulated to produce impulses. The phenomenon will now be illustrated with the use of records obtained with a nerve that had been kept in choline chloride; it must be emphasized, however, that similar phenomena have been observed with nerves kept in the other sodium-free solutions mentioned above (cf. section 10,a).

In the nerve used to obtain the records of figure 13 impulses cou'd be initiated in A fibers in response to the closure of 40 μa currents (record *13;* note the small spike at the start of the applied pulse). With the unconditioned nerve break responses were not elicited by the anodal current (records *14, 15*) but 3 seconds after the end of a 3-second period of anodal polarization brief anodal pulses elicited a break response (record *16*) that increased after the 3-second pulse used to obtain records *17, 18* (record *19*), to become maximal after the 3-second

period of records *21*, *22* (record *23*). The increase in the anodal break response was accompanied by a decrease in the cathodal make response (records *20*, *24*). This phenomenon, post-anodal depression (cf. '47, sections VIII.8 and XV.5c), was a proof that the E_3 reaction was being produced during the establishment of the maximum of the anelectrotonus (records *3*, *7*, *11*) and that, although the electrotonic potential failed to reverse its sign after the end of the polarization (records *4*, *8*, *12*) a change in the relative values of the fractions of the membrane potential had been produced by the reaction, which restored to the nerve fibers the ability to respond to the opening of the anodal current (cf. below, section 9,c).

The fact that with the unconditioned nerve make responses were elicited only by large currents, i.e., by currents 30–40 times greater than the rheobase of normal nerve, readily explains why the impulses failed to propagate themselves along the nerve. The threshold of stimulation was so high that the action current was insufficient to effect restimulation.

Records *1* and *2* of figure 15 show that at the time when all the A fibers had lost their ability to conduct impulses, a large number of fibers of slow conduction still were able to conduct impulses, even though at a reduced speed. During the following hours both the number of responding fibers and the speed of conduction decreased progressively (records *3* to *8*). Finally, 25 minutes after the number of conducting fibers and the speed of conduction had decreased to the extent indicated by records *13* to *15* the nerve was placed in contact with a 0.11 M solution of ion IX, i.e., of the ethyl homologue of choline. Substitution of the methyl groups of choline by ethyl groups produced the expected result (cf. figs. 11 and 12). A large number of fibers of the Et class rapidly regained their abillty to conduct impulses (records, *17* to *24*), even though the restoration was not as complete as that which would have been produced by tetraethyl-ammonium or that which was produced in the companion nerve by n-propyl-triethyl-ammonium (fig. 2, XVI). Records *25* and *26* of figure 15 illustrate the last phases preceding the onset of inexcitability in the companion nerve, that also had been kept in 0.11 M choline chloride, and records *27* to *36* the restoration of excitability. There can be no doubt that in restoring the excitability of Et fibers ion XVI is more effective than ion IX. Thus, the replacement of the three methyl groups of choline by ethyl groups yields an ion (fig. 1, IX) that is able to restore rapidly the excitability of Et fibers, but an even more effective ion is obtained if, in addition, the ethanol group is replaced by an ethyl or a n-propyl group. It will be shown in section 6 that the phenyl group as well as the n-butyl and n-amyl groups also yield ions with three ethyl groups, that are as effective as tetraethyl-ammonium and therefore more effective than ethanol-triethyl-ammonium.

If the nerves are left in choline chloride for several hours after all the nerve fibers have become inexcitable, the restoration of excitability of Et fibers by

quaternary ammonium ions is only partial, even though the restoration by sodium may be complete or nearly so. In the experiment illustrated by figure 16 the restoration was effected in the case of one nerve (fig. 16, *1* to *8*) by means of ion XX (phenyl-triethyl-ammonium), that is known to be even more effective a restoring agent than tetraethyl-ammonium (cf. section 6,b). It can readily be noted in figure 16, *1* to *8* that the restoration of the choline-treated nerve was only partial. In the case of the other nerve (fig. 16, *9* to *15*) the restoration was effected by means of ion XXI (β-phenylethyl-triethyl-ammonium). This ion proved to be less effective than ion XX (cf. section 6,b).

The difference between the effects upon nerve of choline and its ethyl homologue, ethanol-triethyl-ammonium, appears with particular clarity when a comparison is made of the properties of nerves that have been kept in solutions of the two compounds for a long period of time. Figure 17, *1* to *12*, presents the electrotonic potentials recorded from a nerve that had been kept in a 0.11 M solution of ethanol-triethyl-ammonium for 17 hours. In contrast with the results obtained with nerves kept in choline chloride (figs. 13 and 14) the cathodal records (*1*, *5*, *9*) of figure 17 display large slow components, indicating that the L fraction of the membrane potential had a large value. The anodal records (*2* to *4*, *6* to *8* and *10* to *12*) present deflections that, except for the lack of overshootings after the end of polarization, are remarkably similar to those which are observed with nerve kept in Ringer's solution. Furthermore, treatment of the nerve with tetraethyl-ammonium produced only a slight change in the electrotonic potentials (fig. 17, *13* to *24*), a result that contrasts sharply with the change in the anelectrotonus recorded in figure 14.

The A fibers of nerves kept in ethanol-triethyl-ammonium chloride become inexcitable in the same manner as in other sodium-free media; the fibers of slow conduction, however, remain excitable for many hours after the effect of the lack of Na^+ ions should have rendered them inexcitable; moreover, the conducted responses have the same characteristics as in nerves restored by or kept in tetraethyl-ammonium: the speed of conduction is very low and the spike duration very long.

Figure 18 presents records obtained in two different experiments. In one case (records *1* to *4*) the nerve had been kept in the 0.11 M solution of ion IX for 18 hours. The conducted response was recorded at two different points of the nerve, the difference between the conduction distances being 22 mm. As can readily be noted the conducted response included two elevations, labelled Et_1 and Et_2; the fastest fibers of the Et_1 group conducted the impulses at 100 mm per second while the speed of conduction of the fastest fibers of the Et_2 group was 25 mm per second. Repetitive stimulation at 10-second intervals resulted in a marked decrease in the speed of conduction, even though the decrease in the spike height was relatively slight.

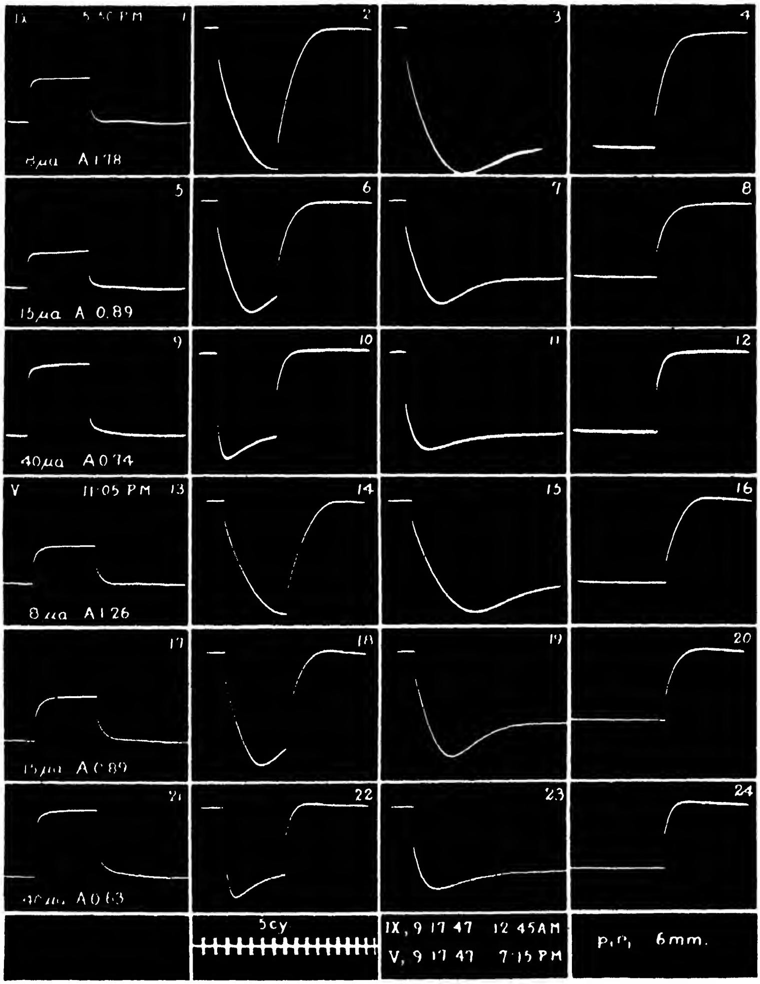
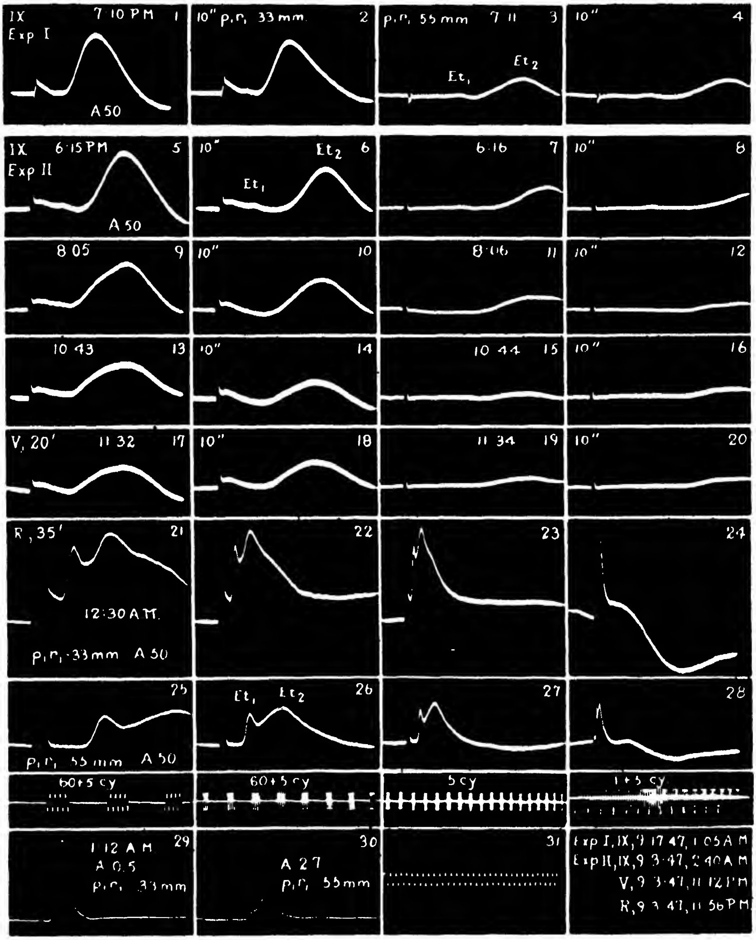


FIG. 17. Electrotonic potentials in a nerve kept in 0.11 M ethanol-triethyl-ammonium chloride for 17 hours (*1* to *12*) and then treated with tetraethyl-ammonium chloride (*13* to *24*).

FIG. 18. Conducted responses in nerves kept in 0.11 M ethanol-triethyl-ammonium chloride.

1 to *4*, observations made in the experiment of figure 17; *5* to *20*, observations made in experiment 9–3–'47, before (*5* to *16*) and after (*17* to *20*) placing the nerve in contact with tetraethyl-ammonium chloride. In both cases the observations were begun after the central segment of the nerve had performed a successful recovery in Ringer's solution. The records of the two columns on the left were obtained with a conduction distance of 33 mm, those of the two columns on the right with a conduction distance



of 55 mm. The intervals of time between successive records (*1*, *2*; *3*, *4*, etc.) were 10 seconds long. Et_1 and Et_2, elevations of the Et spike; note that in the case of records *5* to *20* the speed of conducton of the Et_1 elevation decreased relatively more than that of the Et_2 elevation, so that ultimately the two elevations became fused. The time line below record *27* applies to records *1* to *20*.

21 to *28*, the Et spike, after restoration by Ringer's solution, recorded with two conduction distances (*21* to *24* and *25* to *28*) and 4 sweep speeds (cf. time lines below records *25* to *28*). The Et_1 and Et_2 elevations in record *26* correspond to the Et_1 and Et_2 elevations in records *3* and *6*. Note the large positive after-potential in records *24* and *28*.

29, *30*, the A spike at two different points of the nerve after restoration by sodium. Note the low speed of conduction (cf. record *31*, 1000 cy) and the atypical elevations in the spike.

In the other case the observations were begun after the nerve had been 15½ hours in the solution of ion IX (records *5* to *8*). The conducted response also included two elevations, but the speeds of conduction were lower than in the preceding case (cf. end of section 2). In the continuation of the experiment the height of the conducted response and the speed of conduction were observed to decrease progressively (records *9* to *16*); in particular, it was noted that the Et_1 elevation became fused with the Et_2 elevation, to yield the type of conducted response, which is customarily observed after restoration by tetraethyl-ammonium of nerves that have become inexcitable in solutions of compounds I or II (cf. section 10,c).

At 11:12 P.M. the nerve was treated with 0.11 M tetraethyl-ammonium chloride, which did not prevent the decrease of the conducted response (records *17* to *20*). This result was only an example of a general rule. If the conducted response of nerves kept in solutions of compounds having three ethyl groups, or of nerves that have been restored by compounds having three ethyl groups, begins to decrease spontaneously no improvement of the state of the Et fibers will be obtained with tetraethyl-ammonium, even though the Et fibers would have remained excitable for many additional hours if the nerve had been kept in tetraethyl-ammonium all the time or if the restoration had been effected directly by tetraethyl-ammonium. Apparently, the decrease in the response is referable to the production by compounds having three ethyl groups of a change in the properties of the Et fibers which is not reversible by tetraethyl-ammonium and which is not produced or produced only at an exceedingly low rate by tetraethyl-ammonium itself. The change, however, is reversible by sodium.

In the experiment that is now under consideration the peripheral segment of the nerve was treated with Ringer's solution at 11:56 P.M. As is customarily observed in experiments of long duration the restoration by sodium proved to be a long lasting process. Recovery of the fibers of slow conduction occurred first (records *21* to *28*); later, also the fibers of fast conduction began to recover their ability to conduct impulses (records *29*, *30*). Detailed analysis of records *21* to *30* will be made in section 10.

c. Effect of the acetylation of ethanol-triethyl-ammonium. In view of the well-known fact that acetylation of the alcohol group of choline modifies the properties of the substance, and markedly increases the action of choline upon certain biological systems, it seemed desirable to investigate the effect of the acetylation of the alcohol group in the ethyl homologue of choline. Remarkably enough acetylation of the alcohol group of ion IX yields an ion (fig. 2, XXIV) that is considerably less effective in restoring the excitability of Et fibers deprived of sodium than ion IX itself.

The records reproduced in figures 19 and 20 were obtained in an experiment designed with the purpose of illustrating in clear manner the difference in the

effectiveness of ions IX and XXIV. Two nerves taken from the same frog were kept in a 0.11 M solution of ion X for 13 hours, and after the central segments had performed their recovery in Ringer's solution, one of the nerves

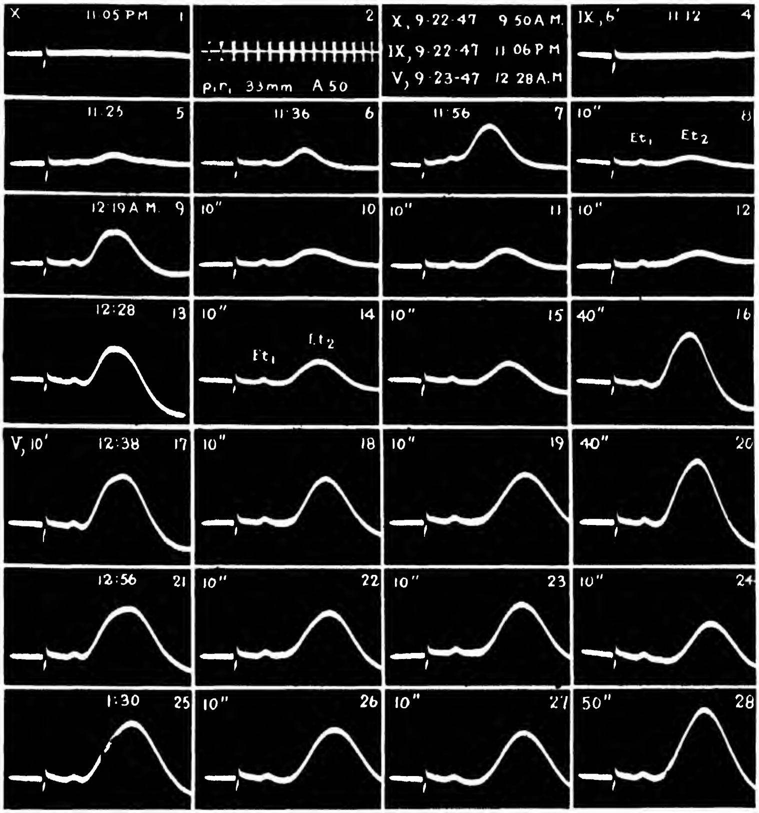


FIG. 19. Restoration of the excitability of the peripheral segment of a nerve rendered inexcitable in a 0.11 M solution of diethanol-dimethyl-ammonium chloride. *1*, absence of response in the peripheral segment; *4* to *16*, restoration by ethanol-triethyl-ammonium ions; *17* to *28*, enhancement of the restoration by tetraethyl-ammonium ions.

was treated with ion IX (fig. 19) and the other with ion XXIV (fig. 20). Records *1* to *16* of figure 19 prove that ion IX was able to effect a far reaching restoration of Et fibers, that was later markedly enhanced by tetraethyl-ammonium (fig. 19, *17* to *28*). In contrast with this result, it is shown by records *5* to *12* of figure 20 that the acetylated ion XXIV was able to restore the ability to conduct impulses to only a small number of Et fibers. The nerve was then

treated with phenyl-triethyl-ammonium, that, be it repeated, is a very effective restoring ion. As a proof that ion XXIV had not produced irreversible damage to the nerve fibers, records *13* to *16* of figure 20 show that in 8 minutes phenyl-

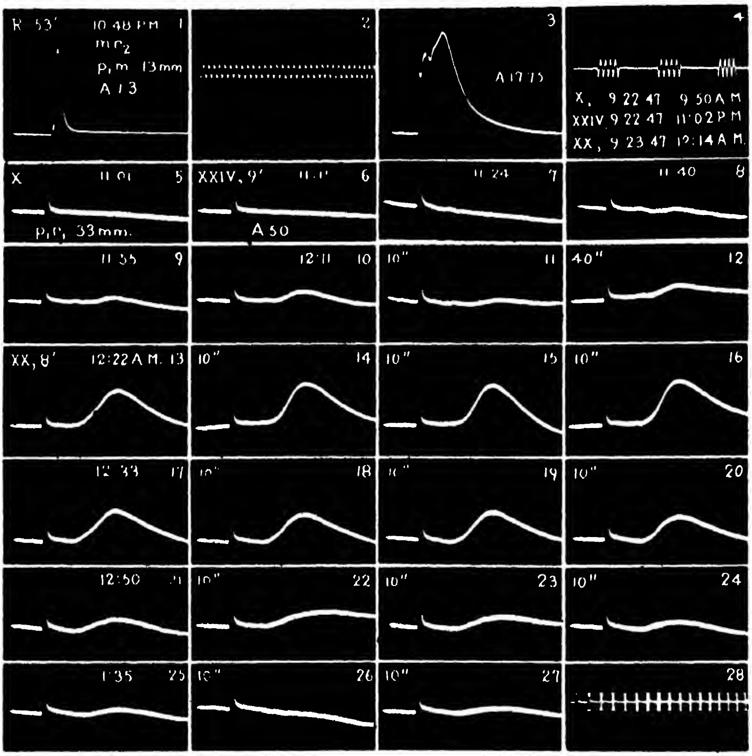


FIG. 20. Restoration of the excitability of a nerve rendered inexcitable in 0.11 M diethanol-dimethyl-ammonium chloride (from the same experiment as fig. 19).

1 and *3*, responses recorded at point m after restoration of the central segment by Ringer's solution; time line *2* (1000 cy) applies to record *1*; time line *4* (60 + 5 cy), to record *2*.

5, absence of conducted response in the peripheral segment of the nerve; *6* to *12*, restoration by acetyl-β-hydroxyethyl-triethyl-ammonium ions (fig. 2, XXIV); *13* to *27*, enhancement of the restoration by phenyl-triethyl-ammonium ions. The conduction distance (33 mm), the amplification (A50) and the sweep speed (record *28*) were constant for records *5* to *27*, and equal to those which were used for the records of figure 19.

triethyl-ammonium was able to restore to a large number of fibers the ability to conduct impulses at 10-second intervals with but little fatigue. The conducted response was later observed to decrease progressively in size (records *17*

to *27*), but this change is known to be a characteristic trait of the action of phenyl-triethyl-ammonium (cf. section 6,b).

A few remarks may be made here. Acetylation of the alcohol group of choline results in a very large increase of the action of the substance upon certain biological systems, such as, for example, the heart, the neuro-muscular junction, the sympathetic synapse, etc. This fact together with other evidence has served to develop concepts on the rôle of acetylcholine in the transmission of nervous effects (cf. Loevi, '33; Dale, '37). Certain aspects of the problem still need clarification; nevertheless, there can be no doubt on the legitimacy of the belief that acetylcholine plays a rôle in the function of those systems upon which acetylcholine has a marked effect.

The situation in the case of nerve is entirely different. Acetylation of the alcohol group of choline does not increase the action of choline upon nerve, since acetylcholine has proved to have no readily detectable action upon nerve (cf. '44 and '47, section IV.7). In addition, it appears now that acetylation of the alcohol group of the ethyl homologue of choline (fig. 1, IX) decreases the restoring action of the substance upon nerve deprived of sodium. From this point of view it appears that acetylation of the ethanol group is not a general mechanism of increase of activity of quaternary ammonium ions. From a more general point of view the fact that acetylcholine (fig. 2, XXV) has no readily detectable effect upon nerve, while many other quaternary ammonium ions have marked effects, this fact appears to be a clear sign of the danger that would be involved in generalizations leading to the assumption that acetylcholine plays a direct rôle in the propagation of the nerve impulse along nerve fibers.

d. Effects of ions VII and VIII. If one of the methyl groups of choline is replaced by the ethyl group, the resulting ion (fig. 1, VII) has properties that resemble those of ions I and II more than the properties of choline. As is shown by records *1* to *12* of figure 21 the electrotonic potentials of nerves kept in a 0.11 M solution of ion VII resemble, rather closely, the electrotonic potentials of nerves kept in a solution of ion I (fig. 4) or of ion II (fig. 7). There is, however, a significant difference between the effects of ion VII and the effects of ions I and II. Tetraethyl-ammonium is able to increase the L fraction (fig. 21, *13*, *17*, *21*) as well as the polarizability of the membrane of nerves kept in a solution of ion VII, but the facts (1) that in records *14*, *15* and *18*, *19* the rate of increase of the anelectrotonus is lower and (2) that the maxima are less pronounced than in the corresponding records of figures 4 and 8, indicate that tetraethyl-ammonium is less effective in restoring nerves treated with ion VII than in restoring nerves kept in solutions of ions I or II. The reason for this difference probably is that ion VII exerts a slight depolarizing action upon nerve (fig. 39, *2*). On the other hand, tetraethyl-ammonium is able to produce only a partial restoration of the ability to conduct impulses

by Et fibers of nerves that have become inexcitable in a solution of ion VII; an irreversible change, however, is not produced by ion VII, since sodium may enable the nerve fibers to perform a successful recovery, even after they have performed an incomplete recovery in tetraethyl-ammonium.

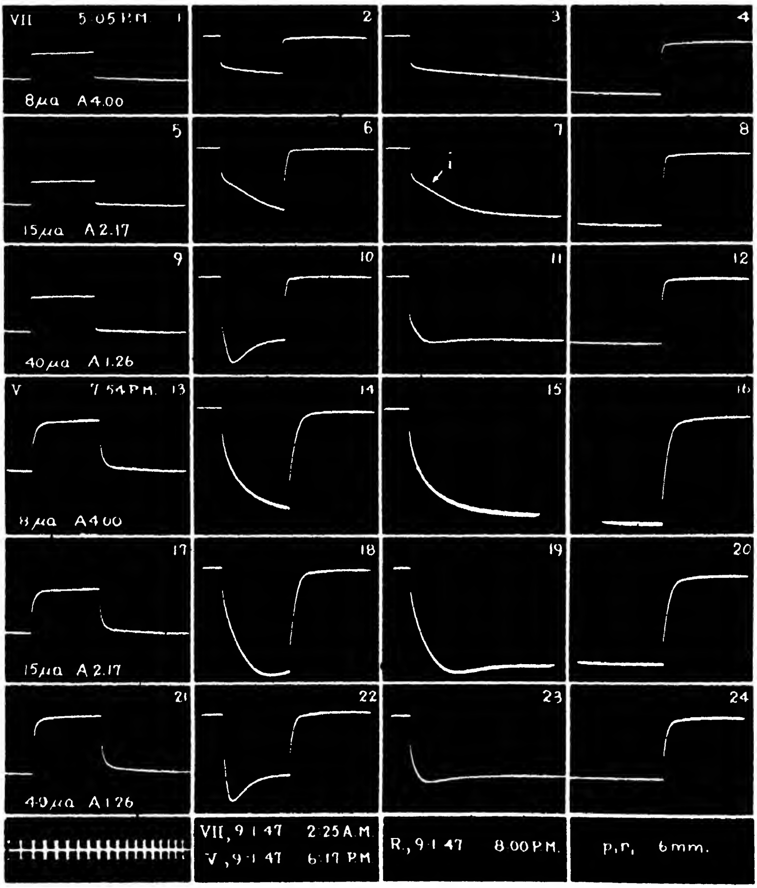


Fig. 21. Electrotonic potentials in a nerve kept in 0.11 M ethanol-dimethyl-ethyl-ammonium chloride, before (*1* to *12*) and after restoration by tetraethyl-ammonium ions (*13* to *24*).

Figure 22 illustrates the results of two different experiments. In both cases the nerves were treated with 0.11 M tetraethyl-ammonium after they had been in the solution of ion VII for practically 15 hours. The recovery of Et fibers began rapidly, since an important number of fibers were found to be able to conduct impulses after 8 minutes in one case (record *3*) and after

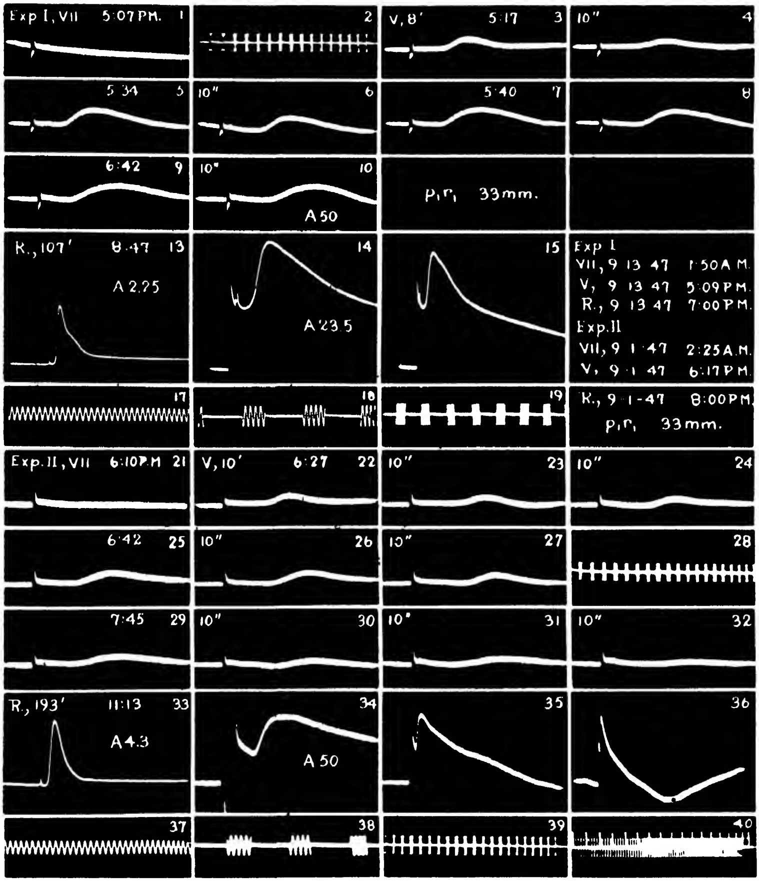


FIG. 22. Restoration of the excitability of the peripheral segments of nerves kept in 0.11 M ethanol-dimethyl-ethyl-ammonium chloride. *1* to *15*, experiment 9–13–'47; *21* to *36*, experiment 9–1–'47 (fig. 21).

1 and *21*, absence of conducted responses in the peripheral segments of the nerves; *3* to *10* and *22* to *32*, restoration of excitability by tetraethyl-ammonium ions. The amplification (A50) and the sweep speed (records *2* and *28*) were constant.

13 to *15* and *33* to *36*, responses after restoration by Ringer's solution; *13* and *33*, A fibers; *14*, *15* and *34* to *36*, fibers of slow conduction. Note (1) the absence of fractionation of the spike, (2) the large negative after-potential in records *14*, *15* and *34*, *35* and the large positive after-potential in record *36*. Time lines *17* to *19* and *37* to *40* apply each to the corresponding record.

10 minutes in the other (record *22*); soon, however, the number of responding fibers ceased to increase (records *5* to *8* and *25* to *27*) and after tetraethyl-ammonium ions had acted upon the nerve for one hour the conducted response

began to decrease (records *9*, *10* and *29* to 32). The action of sodium resulted in a far reaching recovery; at the times when records *13* to *15* and *33* to *36* were obtained a large fraction of the A fibers and probably all the fibers of slow conduction were able to conduct impulses. An important detail is this: although tetraethyl-ammonium had been able to restore the ability to conduct impulses to only a fraction of the Et fibers, the shape and duration of the spikes reproduced in records *14*, *15* and *34* to *36* prove that tetraethyl-ammonium had produced a characteristic change in the properties of all the fibers of slow conduction (cf. section 10).

A radical change in the properties of choline results from the substitution of two methyl by two ethyl groups. As described in section 3,b, ion VIII can effect a restoration of the excitability of certain fibers of class Et after these fibers have become inexcitable in a solution of ion II. In sharp contrast with this result nerves kept in a 0.11 M solution of ion VIII become irreversibly inexcitable; indeed, the deleterious effect of ion VIII goes so far that the nerve fibers lose their core conductor properties. In this respect the properties of compounds IV and VIII are identical; also, it will be shown in section 8 that both ions are powerful depolarizing agents (cf. fig. 38, *4*, *5*; fig. 39, *3*).

At the start of the observations illustrated by figure 23, *1* to *8* the nerve had been kept in a 0.11 M solution of ion VIII for 15 hours. The electrotonic potentials produced by applied currents (records *1* to *6*) were exceedingly small; indeed, they would have been almost undetectable at the amplifications used to obtain the records of figures 4, 7, 9, 13, 14, 17 and 21; moreover, the electrotonic potentials appeared as rectangular deflections, such as are observed with nerves that have undergone a far reaching depolarization, for example, as a result of the action of ether at a high concentration (cf. '47, section VII.1). The magnitude and temporal course of the electrotonic potentials indicated that the nerve fibers had undergone severe damage, and in confirmation of this conclusion it was found that treatment of the nerve with Ringer's solution failed to prevent further deterioration of the nerve fibers. Thus, at the time when records *7* and *8* were obtained the nerve fibers had already lost entirely their core conductor properties, and for this reason no electrotonic potentials were produced by the applied current.

In the experiment illustrated by figure 23, *10* to *18* the nerve was kept in the 0.11 M solution of ion VIII for 12½ hours. Accordingly, the deterioration of the nerve fibers was not as far advanced as in the experiment just described, and after the nerve had been treated with Ringer's solution a partial recovery took place. Remarkably enough, the fibers that regained their ability to conduct impulses (fig. 23, *10*, *13*, *16*) were fibers of the A group; no fiber of slow conduction recovered its excitability, since records *11*, *14* and *17* of figure 23 present only the electrotonic potentials produced by the stimulating current.

e. Comment. The results presented in this section again emphasize how

radical may be the change in properties of quaternary ammonium ions caused by substitution of the groups attached to the nitrogen nucleus. One may be tempted to attach particular significance to the ions of the choline group,

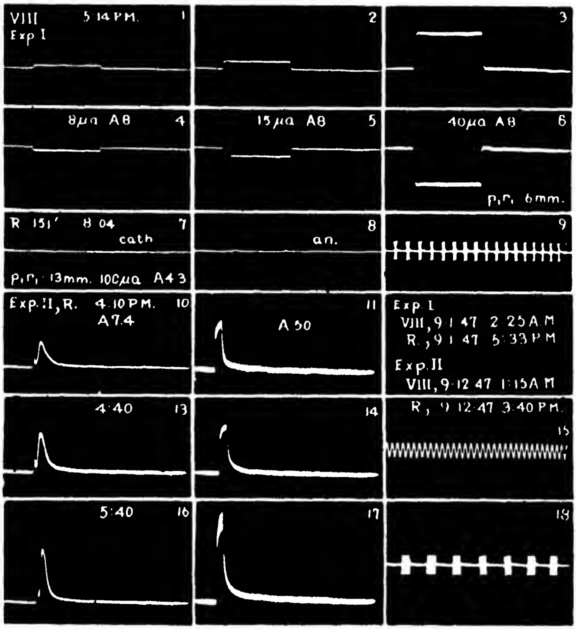


FIG. 23. Observations made with nerves kept in 0.11 M ethanol-methyl-diethyl-ammonium chloride.

1 to *6*, electrotonic potentials produced by currents of three different magnitudes, at 6 mm from the polarizing electrode; note the absence of slow components and the small size of the deflections; *7*, *8* absence of electrotonic potential at 13 mm from the polarizing electrode after the nerve fibers had lost their core conductor properties. Record *7* was obtained with the use of the cathodal current; record *8*, with the use of the anodal current (experiment 9–1–'47).

10 to *17*, restoration of the excitability of the central segment of the nerve in experiment 9–12–'47; *10, 13, 16*, restoration of A fibers; *11, 14, 17*, lack of restoration of fibers of slow conduction. The deflections in records *11, 14, 17*, are the electrotonic potential produced in the A fibers of the stimulating current (100 μa, records *11*, *14*; 200 μa, record *17*); note the difference between records *7*, *8*, and *11, 14, 17*. Time line *15* applies to records *10, 13, 16*; time line *18*, to records *11, 14, 17*.

because choline and acetylcholine are known to be constituents of nervous tissue; as a matter of fact, however, present day knowledge of the quarternary ammonium compounds occurring in nervous tissue is exceedingly limited.

5. *Ions with Two or More Ethanol Groups*

A study is made in this section of the action upon nerve of the quarternary ammonium ions listed in rows 3, 4 and 5 of figure 1.

a. Ions X (diethanol-dimethyl-ammonium) and XI (diethanol-methyl-ethyl-ammonium). Ion X results from the substitution in choline of an ethanol for a methyl group. The substitution modifies the properties of choline in a remarkable manner, since nerves kept in a solution of ion X instead of behaving like nerves kept in a solution of choline behave like nerves kept in a solution of tetramethyl-ammonium (ion I). Unlike choline, ions I and X do not produce in the nerve fibers changes that are reversible only with difficulty or not reversible by quaternary ammonium ions having three or more ethyl groups. For this reason 0.11 M solutions of compounds I or X are the best available media for the study of the effect upon nerve of the lack of sodium ions; as a matter of fact, although the difference can be only slight, ion X seems to be even more "inert" than compound I, since loss of the ability to conduct impulses by all the fibers of the nerve takes place somewhat more rapidly in a solution of ion X than in a solution of ion I. For this reason, after the properties of ion X became known, the experiments have been done routinely with nerves that had become inexcitable in a 0.11 M solution of ion X. On the other hand, the chloride of diethanol-dimethyl-ammonium can be prepared in a state of great purity very easily.

Figure 24 illustrates the electrotonic potentials that are observed with nerves that have become inexcitable in a 0.11 M solution of ion X (records *1* to *8*) and the changes in the value of the L fraction of the membrane potential and in the polarizability of the membrane that result from treating the nerve with tetraethyl-ammonium chloride (records *9* to *16*) and with sodium chloride (records *17* to *24*). It can readily be noted that the changes are essentially identical with those recorded in figures 4, 7 and 8. A more detailed analysis will be made in section 9.

Examples of restoration of nerves rendered inexcitable in solutions of ion X by solutions of other quaternary ammonium ions and by sodium are presented in figures 19, 20 and many others.

Substitution in ion X of one methyl group by one ethyl group results in a radical change in properties, since ion XI produces irreversible deterioration of the nerve fibers. In this respect ion XI acts like ions VIII and IV; as will be shown in section 8 all three ions, XI, VIII and IV are powerful depolarizing agents.

In the experiment illustrated by figure 25 the electrotonic potentials were recorded in the peripheral segment of the nerve after the 0.11 M solution of ion XI had acted upon the nerve for 15½ hours. Both the catelectrotonus (records *1* to *3*) and the anelectrotonus (records *4* to *6*) had small magnitude and what is more significant, the slow component of the anelectrotonus was

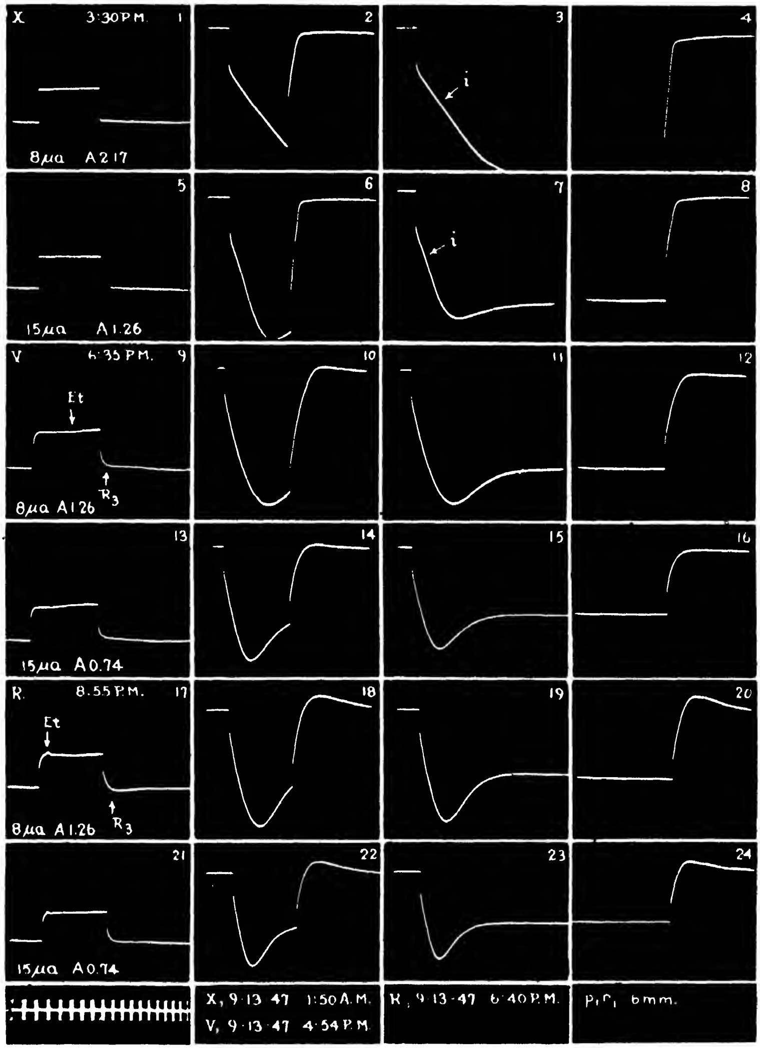


FIG. 24. Electrotonic potentials produced in a nerve kept in 0.11 M diethanol-dimethyl-ammonium chloride, before (*1* to *8*) and after restoration by tetraethyl-ammonium chloride (*9* to *16*) and by Ringer's solution (*17* to *24*). R_3, overshooting of the slow catelectrotonus; Et, spikes of fibers of slow conduction; note the difference in the speed of conduction in records *9* to *17*; note also the difference in the spike duration, in the case of record *9* the applied pulse was interrupted at the time when the spike was reaching its crest. Among the fibers of slow conduction only low threshold B fibers can be stimulated by the 8 μa current.

very small even in the case of the 40 μa current (record *6*). The magnitude and the temporal course of the electrotonic potentials were clear signs of the precarious condition of the nerve fibers. The deterioration increased with advancing time and 4 hours after records *1* to *6* had been obtained the nerve fibers had practically lost their core conductor properties, as is shown by the fact

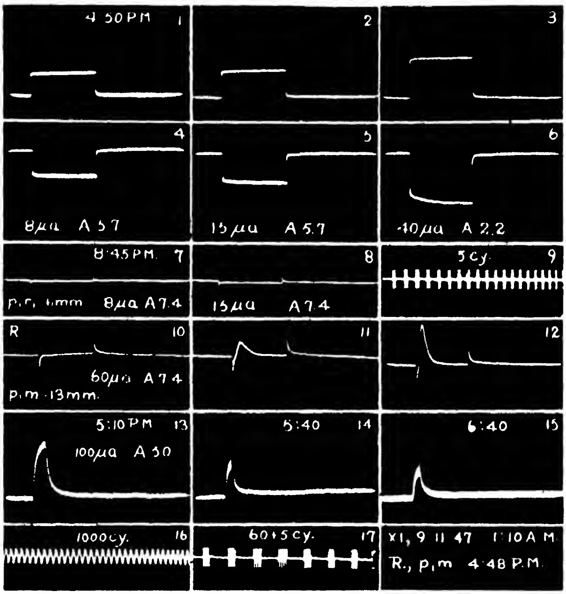


FIG. 25. Illustration of the effect of diethanol-methyl-ethyl-ammonium ions.

1 to *6*, electrotonic potentials produced by currents of three different magnitudes, before the nerve fibers had lost their core conductor properties; note the small size of the deflections. *7*, *8*, absence of electrotonic potentials after the nerve fibers had lost their core conductor properties; records *7* and *8* were obtained with the use of anodal currents.

10 to *15*, effect of Ringer's solution upon the central segment of the nerve; *10* to *12*, restoration of the excitability of a few A fibers; *13* to *15*, absence of restoration of fibers of slow conduction. A 200 μa current was used for record *13*; 100 μa currents, for records *14* and *15*.

that the electrotonic potentials that appear in records *4* and *5* became those which appear in records *7* and *8*.

In order to ascertain the degree of reversibility of the action of ion XI the central segment of the nerve was placed in contact with Ringer's solution at 4:48 P.M., i.e., immediately before records *1* to *6* had been obtained. A small number of fibers of the A group recovered their ability to conduct impulses (records *10* to *12*); no fiber of slow conduction, however, became ex-

citable, since records *13* to *15* present only the electrotonic potentials produced by the stimulating current. Thus, there is the remarkable fact, that ion XI like ions VIII and IV causes irreversible deterioration of all the fibers of slow conduction earlier than it causes irreversible deterioration of all the A fibers.

***b. Ions XIII** (triethanol-methyl-ammonium), **XIV** (triethanol-ethyl-ammonium) **and XV** (tetraethanol-ammonium).* If in ion X a methyl group is replaced

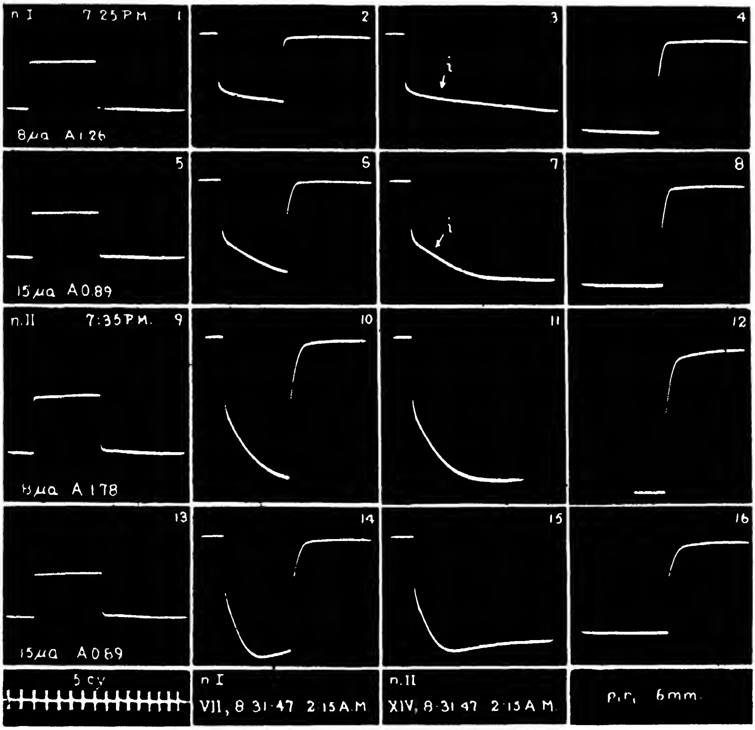


FIG. 26. Electrotonic potentials of nerves kept in ethanol-dimethyl-ethyl-ammonium chloride (*1* to *8*) and in triethanol-ethyl-ammonium chloride (*9* to *16*).

by an ethanol group there results ion XIII, that has properties closely resembling those of ion VII. For this reason there is no need of illustrating the properties of ion XIII. It will be convenient, however, to study ion XIV, that results from substituting an ethanol for the methyl group in compound XI. The important difference that exists between the properties of ions VII and XIV appears with particular clarity when a comparison of the two ions is done with the use of paired nerves.

Figure 26 presents the electrotonic potentials recorded from two nerves, one of which (records *1* to *8*) had been kept in a 0.11 M solution of ion VII for 17

hours, and the other in a 0.11 M solution of ion XIV for the same length of time. As can readily be noted, both ions had produced the same qualitative changes in the electrotonic potentials, but the polarizability of the membrane was greater in the nerve kept in the solution of ion XIV than in the nerve kept in the solution of ion VII.

After the central segments of the two nerves had performed their recovery in Ringer's solution it was found that while no fiber of the nerve kept in the solution of ion VII was able to conduct impulses into the peripheral segment (fig. 27, *1*) a number of fibers of slow conduction of the nerve kept in the solution of ion XIV was able to conduct impulses (fig. 27, *17*, *18*), with the noteworthy peculiarity that these fibers conducted at the low speed which is so characteristic for nerves restored by quaternary ammonium ions with three or 4 ethyl groups (cf. below, section 10). Ion IX restored the ability to conduct to only a small number of Et fibers of the first nerve (fig. 27, *2* to *4*), a predictable result since even tetraethyl-ammonium would have been able to restore only a fraction of the Et fibers (fig. 22). The remarkable observation was that tetraethyl-ammonium was able to increase the number of conducting fibers of the second nerve only by a small amount (fig. 27, *19* to *22*). After the response had reached the size with which it appears in records *21*, *22*, it decreased progressively (records *23* to *25*). This result is an example of the general rule that was mentioned in section 4,b.

Sodium produced a far reaching restoration of both nerves. Records *9* to *12* illustrate late phases of the restoration of the fibers of fast conduction (records *9* and *11*) and of slow conduction (records *10* and *12*) of the nerve that had been kept in the solution of compound VII. The initial phases of the restoration by sodium of the nerve kept in the solution of compound XIV are illustrated by records *26* to *29;* it will be noted that the restoration was a slow process. No A fibers had become able to conduct impulses at the time when record *29* was obtained, but later the restoration of A fibers (record *30*) and of fibers of slow conduction (records *31* and *32*) became complete.

The properties of ion XV resemble those of ion XIV except in one detail. The fibers of slow conduction became inexcitable in a solution of ion XV not later than in a solution of ion I or X. An illustration of the effect of ion XV on the electrotonic potentials will be presented in section 9,c.

c. Comparison of ions III (dimethyl-diethyl-ammonium) and XII (diethanol-diethyl-ammonium). Particular attention deserves a comparison of the properties of ions III and XII. The fact that fibers of class Et remain able to conduct impulses longer in solutions of ion XIV than in solutions of ions II or VII indicates that in a quaternary ammonium ion the ethanol group may play a rôle akin to that which is played by the ethyl group. If this were the case the properties of ion XII should resemble the properties of tetraethyl-ammonium more than the properties of ion III. This is, indeed, the case.

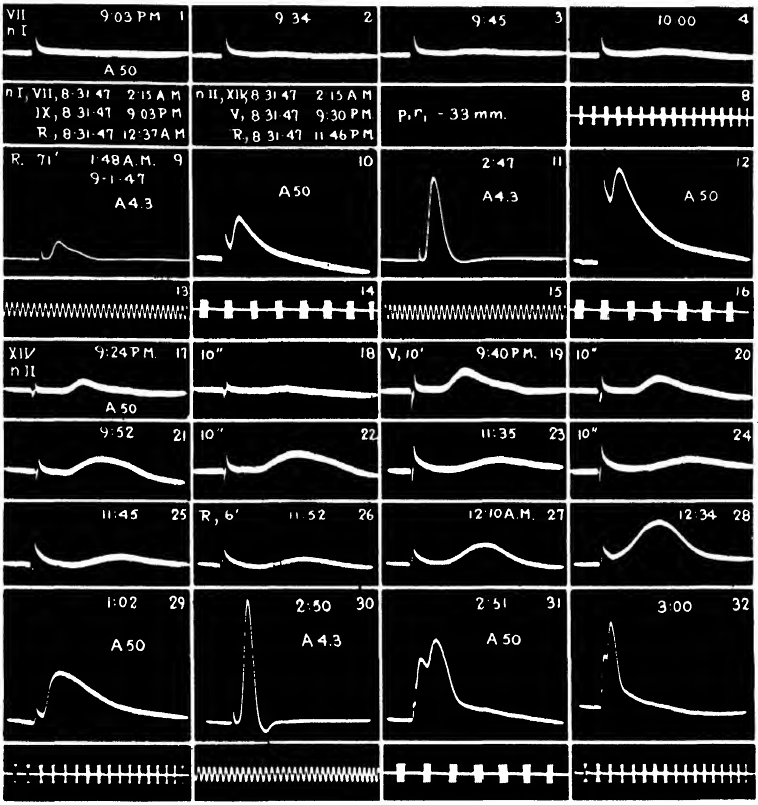


FIG. 27. Illustration of the difference between the effects of ethanol-dimethyl-ethyl-ammonium and triethanol-ethyl-ammonium ions.

1, absence of conducted response in the peripheral segment of nerve I, kept in 0.11 M ethanol-dimethyl-ethyl-ammonium chloride; *2* to *4*, restoration by ethanol-triethyl-ammonium ions; *9* to *12*, restoration by sodium.

17, *18*, conducted responses in the peripheral segment of nerve II, kept in 0.11 M triethanol-ethyl-ammonium chloride; *19* to *25*, restoration by tetraethyl-ammonium ions; *26* to *32*, restoration by sodium. The sweep speed for records *17* to *29* was constant (time line below record *29*).

The experiment illustrated by figures 28 to 30 was done with the use of the two sciatic nerves of a bullfrog. One of the nerves was kept in a 0.11 M solution of ion III for 15 hours and the other in a 0.11 M solution of ion XII for the same length of time. Records *1* to *8* of figure 28 show that in the solution of ion III essentially those changes in the properties of the membrane had occurred which would have taken place if the nerve had been kept in a solution of ion I or of ion II (figs. 4 and 7). Treatment of the nerve with tetra-

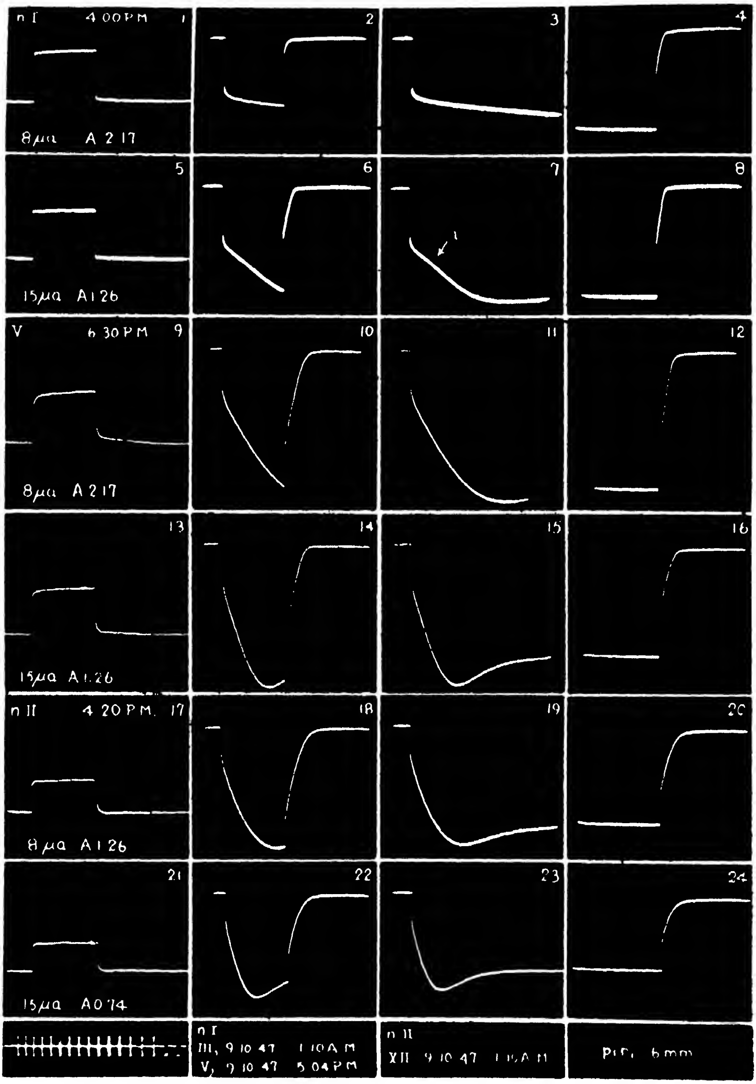


FIG. 28. Illustration of the difference between the effects of dimethyl-diethyl-ammonium and diethanol-diethyl-ammonium ions.

1 to *16*, electrotonic potentials in nerve I, kept in 0.11 M dimethyl-diethyl-ammonium chloride, before (*1* to *8*) and after restoration by tetraethyl-ammonium ions (*9* to *16*).

17 to *24*, electrotonic potentials in nerve II, kept in 0.11 M diethanol-diethyl-ammonium chloride.

ethyl-ammonium produced the expected changes in the electrotonic potentials (records *9* to *16*), with the noteworthy peculiarity that the state of this nerve

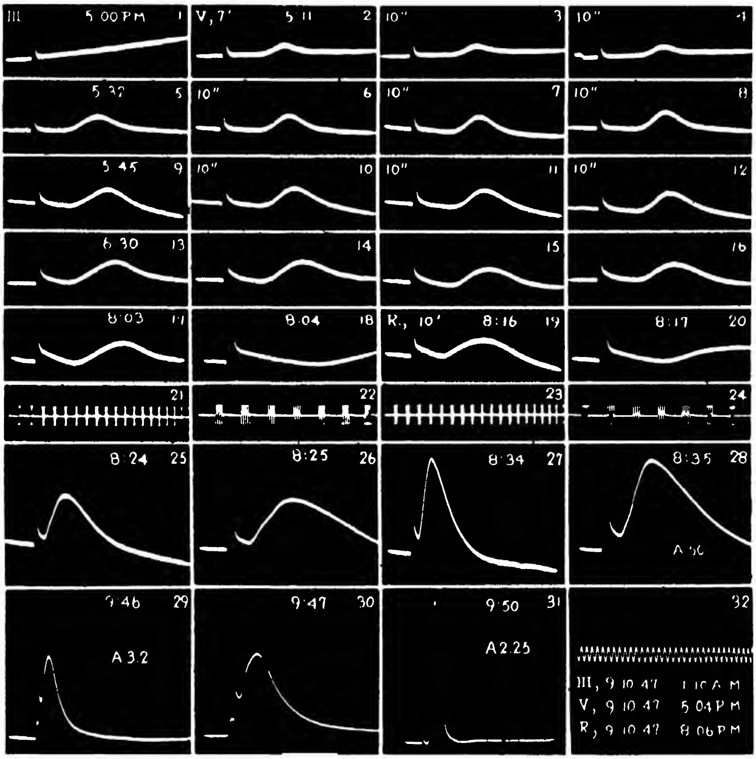


FIG. 29. Restoration of the excitability of the peripheral segment of a nerve rendered inexcitable in 0.11 M dimethyl-diethyl-ammonium chloride.

1, absence of conducted response in the peripheral segment; *2* to *18*, restoration by tetraethyl-ammonium; *19* to *31*, restoration by sodium. The sweep speed was constant for records *1* to *16* (record *21*); records *17* to *30* were obtained at two different sweep speeds (*21*, *23*; *22*, *24*). Time line *32* (1000 cy) applies to record *31*. A3.2 on record *29* should read A32.

became quite similar to the state of the other nerve (records *17* to *24*), that had been kept in the solution of ion XII all the time.

Record *1* of figure 29 shows that all the fibers of the nerve kept in the solution of ion III had lost their ability to conduct impulses. Tetraethyl-ammonium was able to restore rapidly the ability to conduct impulses with but little fatigue to a significant number of fibers (records *2* to *4*); nevertheless, the size of the restored spike increased relatively little during the following 40 minutes

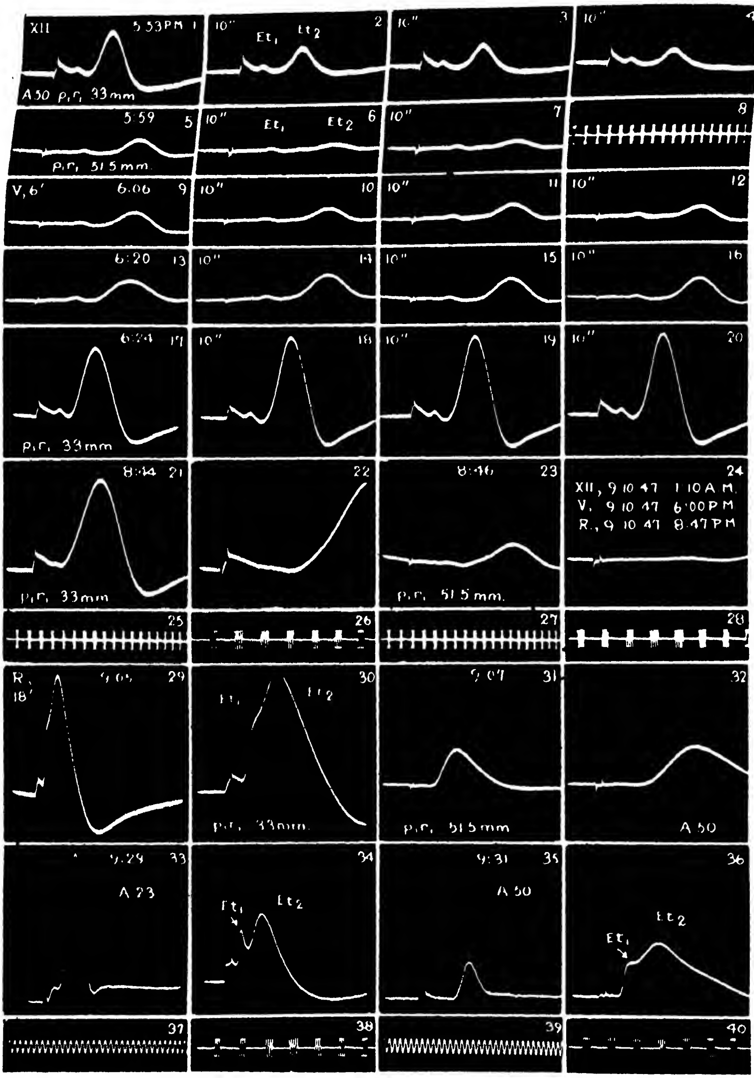


FIG. 30. Improvement of the excitability of the peripheral segment of a nerve kept in 0.11 M diethanol-diethyl-ammonium chloride.

1 to *7*, conducted responses in the peripheral segment of the nerve, recorded with two different conduction distances, 33 mm (*1* to *4*) and 51.5 mm (*5* to *7*). *9* to *24*, increase in the conducted response brought about by tetraethyl-ammonium ions; *29* to *36*, increase in the response brought about by sodium. Records *1* to *20* were obtained with constant sweep speed (*8*); records *21* to *32*, at two different speeds (*25*, *27*; *26*, *28*) and records *33* to *36* at two different speeds (*37*, *39*, 1000 cy; *38*, *40*, 60 + 5 cy). Note the differences in behavior of the Et_1 and Et_2 elevations during repetitive activity (records *1* to *20*) and during the restoration by sodium.

(records *5* to *12*) and later a progressive decrease was observed (records *13* to *18*). In view of the small size of the restored spike in records *9* to *12* and in consideration to the general rule mentioned in section 4,b, a likely assumption would be this. The group of Et fibers that recovers its ability to conduct impulses in solutions of ion III (fig. 10) had undergone changes that were not reversible by tetraethyl-ammonium, so that this ion restored the ability to conduct only to fibers of the rest of the Et class. Sodium, however, proved to be able to restore not only all the fibers of slow conduction (fig. 28, *19* to *30*), but ultimately also the fibers of fast conduction (fig. 28, *31*).

In contrast with the results obtained with the nerve kept in the solution of ion III, many fibers of slow conduction of the nerve kept in the solution of ion XII proved to be able to conduct impulses (fig. 30, *1* to *7*). The number of conducting fibers was not as great as it would have been if the nerve had been kept in a solution of ion IX (fig. 18) but it was quite important; moreover, the shapes of the conducted spikes in figures 18, *1* to *8* and 30, *1* to *7* were quite similar. Tetraethyl-ammonium was able to restore conduction probably to all the fibers of the Et class; it also increased the speed of conduction of the responding fibers (records *9* to *24*). The recovery effected by the action of sodium is illustrated by records *29* to *36*.

d. Comment. From the results presented in this section and in sections 3 and 4 it follows that the 15 quaternary ammonium ions listed in figure 1 can be classified into several groups.

In the first place, ions I, II and X form a group of ions that may be called "inert," since the Et fibers of nerves kept in 0.11 M solutions of those ions lose their excitability without undergoing changes that are not reversible by tetraethyl-ammonium, and since nerves kept in those solutions maintain the total value of their membrane potential at practically the same level as nerves kept in Ringer's solution. Another reason to call ions I, II and X inert is that the properties of nerves kept in 0.11 M solutions of these ions closely resemble the properties of nerves kept in a 0.20 M solution of saccharose containing the chloride of ion I, II, III, VI or X at the concentration 0.01 M or of sodium at the concentration of 0.008 M and of potassium at the concentration 0.005 M.[6] In all probability all the changes that take place in nerves

[6] As was explained elsewhere ('47, Chapter IV) saccharose has no action of its own upon nerve, but the membrane potential of nerves kept in a 0.22 M solution of saccharose with Na^+ ions at the concentration 0.006 M undergoes a progressive increase, that in all probability is referable to the absence of a sufficient number of ions in the external medium of the nerve fibers, since the increase in the membrane potential can be prevented by adding a relatively small number of ions to the solution. On the other hand, nerves kept in solutions of saccharose containing ionized solutes at concentrations below 0.01–0.008 M undergo certain physical changes: after several hours the nerves swell slightly and become translucid, as well as somewhat rigid. These phys-

kept in a 0.11 M solution of one of three inert ions (I, II, X) are referable solely to the lack of Na^+ ions.

Choline (fig. 1, VI) probably has specific properties. Except for certain slight differences that were described in section 4,b nerves kept in a 0.11 M solution of choline chloride behave for a number of hours like nerves kept in the solution of one of the inert ions; later, however, nerves kept in choline undergo changes that are not reversible by tetraethyl-ammonium. Ultimately, nerves kept in choline chloride become inert physicochemical systems having remarkable properties ('47, section VIII.4c); since the observations on nerves kept in solutions of one of the inert ions (I, II, X) have never been continued for more than 30 hours it is not possible to state with certainty whether or not ultimately the inert ions produce the same results as choline; it is certain, however, that they do not do so as early as choline.

The three ions, XI, VIII and IV form an interesting group of active ions. It will be shown in section 8 that these three ions cause a far reaching depolarization of the nerve fibers, and from the results presented in sections 4 and 5 it follows that nerves kept in solutions of those ions undergo irreversible deterioration that may go so far as to cause the loss of core conductor properties of the nerve fibers, i.e., a total disintegration of the membrane. In this respect 0.11 M solution of ions XI, VIII and IV are even more active than 0.11 M solutions of K^+ ions.

That ions XI, VIII and IV are active can have only one explanation, that the ions enter into chemical combination with constituents of the nerve fibers and alter the normal course of those metabolic reactions which underlie the maintenance of both anatomical integrity and functional ability of the nerve fibers. In brief, ions XI, VIII and IV play the rôle of metabolic inhibitors. For this reason it is important to note that ions XI, VIII and IV produce irreversible deterioration of all the fibers of slow conduction at a time when the changes in at least a significant number of fibers of fast conduction still are reversible. This difference undoubtedly is in close relation to the fact that those quaternary ammonium ions listed in figures 1 and 2 which restore the excitability of nerve fibers are able to restore only fibers of slow conduction. As will be remembered ions VIII and IV are two of the restoring ions (figs. 6 and 11).

---

ical changes are not a sign of deterioration of the nerve fibers, since even after 24–36 hours in the saccharose solution the excitability of the nerve can be restored by tetraethyl-ammonium (Et fibers) or by sodium (all fibers). Addition to the solution of saccharose of any chloride, including ammonium and lithium chlorides, at the concentration 0.01–0.008 M is sufficient to prevent those physical changes almost completely; a remarkable fact that deserves detailed study. Potassium or rubidium chloride prevents the physical changes but causes a depolarization of the nerve fibers unless their concentration be reduced to about 0.003 M.

If in examining figure 1 consideration is given to those substitutions of groups which are necessary to pass from one of the three ions, XI, VIII and IV to one of its neighbors or conversely, it becomes immediately obvious that the action of ions XI, VIII and IV cannot be explained in terms of the presence in the ion of a particular group (methyl, ethyl, ethanol) or to the presence of a given number of groups of a certain kind. The activity or rather the reactivity, i.e., the ability of those ions to enter into chemical combination with constituents of the nerve fibers, has to be explained in terms of properties of the ion, that are due to the presence of certain combinations of groups attached to nitrogen. The fact that all these ions contain one methyl group, while the other three groups are combinations of ethyl and ethanol groups may be significant, especially since the action of ion XIII, that also lies in the diagonal 4,d of figure 1, has a certain resemblance with the action of ions XI, VIII, and IV. On the other hand, since the properties of ions VII and XIII are similar, the assumption would be permissible that the 4 ions of diagnonal 4,d (fig. 1) and ion VII form a group of ions with important chemical properties in common.

In regard to the restoration of excitability of nerves deprived of sodium, the 15 ions listed in figure 1 can be classified into two classes, ions with less than two ethyl groups and ions with two or more ethyl groups. Only the ions of the second class have proved to be able to effect restoration. The effectiveness of an ion as a restoring agent can be measured in terms of (1) the rapidity of its action (2) the number of fibers it is able to restore and (3) the length of time that the nerve can be kept in a sodium-free medium before the ion in question becomes unable to effect restoration; this length of time increases with the effectiveness of the ion. By using the three criteria it can be established readily that the effectiveness of the restoring ions increases with the number of ethyl groups that they contain and to a lesser extent with the number of ethanol groups. Thus, the ions of column c, d and e of figure 1 can be arranged in the following series, in which effectiveness increases from left to right: III < VIII < XII ≤ IV < IX < V.

The ability of a quaternary ammonium ion to restore the excitability of nerves deprived of sodium is in no direct relation to the effect of the ion upon the nerve fibers in experiments of long duration. In this respect the comparison of ions IV and IX is particularly instructive. Both ions are able to restore the excitability of Et fibers deprived of sodium, but while nerves kept in a 0.11 M solution of ion IV undergo depolarization and irreversible deterioration, nerves kept in a 0.11 M solution of ion IX maintain their membrane potential at practically the normal level. This result emphasizes the difference that exists between the mechanism that maintains the resting membrane potential of nerve and the mechanism that underlies the establishment of the alteration or nerve impulse (cf. '47, Concluding Notes).

The sharp difference between the properties of ion IV and those of its neighbors (III, V) makes the series I to V discontinuous; a continuous series, however, is formed by the 5 ions of diagonal 5,e of figure 1. In a solution of ion XV the Et fibers become reversibly inexcitable in about the same length of time as in a solution of ion I; but the other 4 ions of diagonal 5,e are able to maintain Et fibers excitable after the effect of the lack of Na^+ should have rendered them inexcitable. The number of excitable Et fibers increases with the number of ethyl groups in the ion.

6. Restoration of Excitability by Quaternary Ammonium Ions with Three Ethyl Groups

This section deals with the effect that a change in the 4th group has upon the ability of an ion with three ethyl groups to restore the excitability of nerve deprived of sodium. The ions that have been used are listed in columns a, b and d of figure 2.

a. Ions listed in columns a and d of figure 2. As already described in section 3,b ion V is more effective than ion IV in restoring the excitability of nerve fibers. The proof of this statement presented in figure 6 is very conclusive. After ion IV had proved to be able to effect only a partial recovery of Et fibers, ion V produced a marked improvement of the recovery. By means of a similar procedure it was shown in the experiment illustrated by figure 11 that ion V is more effective than ion IX; the difference, however, between the effectiveness of ions IX and V is not very great, as should be expected from the similarity of the rôles played by ethyl and ethanol groups in quaternary ammonium ions (cf. section 5,c and d).

The substitution of one of the ethyl groups of ion V by the n-propyl group yields compound XVI, the properties of which, as a restoring agent, are exceedingly similar to those of ion V. Indeed, in order to detect a clear difference between the properties of ions V and XVI it is necessary to determine the changes in the membrane potential that result from long lasting action of the two ions (section 8). Figure 15, *25* to *36* illustrates the effectiveness with which ion XVI restored the excitability of a nerve that had been kept in a 0.11 M solution of choline chloride.

If the n-butyl group is substituted for one of the ethyl groups of tetraethyl-ammonium, the resulting ion (XVII) also is very effective in restoring the excitability of Et fibers. Its restoring ability is somewhat smaller than that of tetraethyl-ammonium, since the recovery of Et fibers begins a few minutes later with ion XVII than with ion V; ultimately, however, the spike height becomes practically as great with ion XVII as with ion V. Thus, no significant error can be committed in stating that the effectiveness of ions V and XVII are practically equal.

This result is, from a certain point of view, important. As was ascertained

in section 4,c ion XXIV is considerably less effective than ion IX and consequently also less effective than ion V. Since the 4th groups in ions XVII and XXIV have the same number of carbon atoms it is clear that lengthening of the carbon chain cannot be the direct cause of the reduction of the effectiveness of ion IX, which results from the acetylation of the alcohol group.

Lengthening of the carbon chain of the 4th group in ion V ultimately results in a significant reduction of the restoring ability of the ion. Probably this result is in direct connection with the observation that lengthening of the chain increases the depolarizing action of the ion (cf. section 8). Ion XVIII (n-amyl-triethyl-ammonium) still has a restoring action only slightly weaker than that of tetraethyl-ammonium, but the restoring action of ion XIX (n-hexyl-triethyl-ammonium) is quite small.

The experiment illustrated by figures 31 and 32 was done with the use of paired nerves that were rendered inexcitable by their being kept in a 0.11 M solution of ion X for 16½ hours. After the central segments of the two nerves had performed successful recoveries in Ringer's solution, the peripheral segment of one of the nerves (fig. 31) was treated with a 0.11 M solution of n-amyl-triethyl-ammonium chloride (fig. 2, XVIII) and that of the other nerve (fig. 32) with a 0.11 M solution of n-hexyl-triethyl-ammonium chloride (fig. 2, XIX).

The recovery of the nerve treated with n-amyl-triethyl-ammonium ions (fig. 31, *3* to *24*) began somewhat later than it would have begun if the nerve had been treated with tetraethyl-ammonium ions, since no conducted response was observed after 10 minutes of action of ion XVIII (record *3*) and even after 22 minutes the conducted response was quite small (record *4*); nevertheless, the recovery progressed steadily with advancing time and after two hours the response restored by n-amyl-triethyl-ammonium ions became as great as that which would have been restored by tetraethyl-ammonium ions (records *5* to *20*). That the recovery of the Et fibers ultimately had become complete is shown by the fact that treatment of the nerve with sodium produced an increase in the speed conduction but hardly any increase in the number of conducting fibers (cf. records *17* and *22*). At the time when the observations were discontinued the fibers of fast conduction were beginning to recover their ability to conduct impulses (record *21*); on the basis of the results of other similar experiments it may be stated with assurance that the recovery of the A fibers would have become complete.

The recovery of the Et fibers of the nerve treated with n-hexyl-triethyl-ammonium chloride (fig. 32) was only incomplete. Record *4* shows that 12 minutes after the solution of ion XIX had been placed in contact with the nerve no nerve fiber had recovered its ability to conduct impulses; the recovery, however, began shortly thereafter and soon the response reached its maximal height (records *5* to *8* and *9* to *12*). Then, the response decreased progressively (records *13* to *20*), which was referable to the production of an irreversible

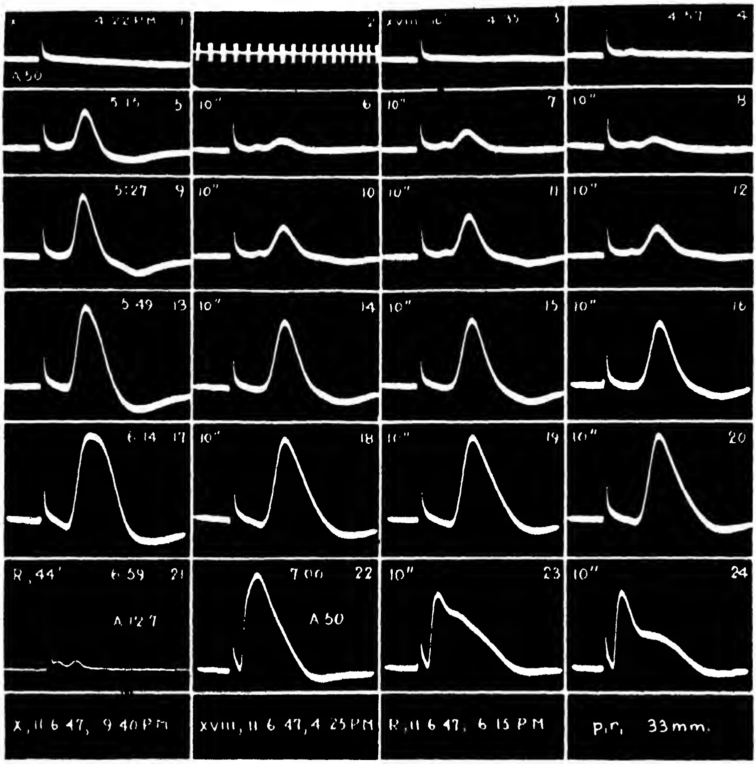


FIG. 31. Restoration of the excitability of the peripheral segment of a nerve rendered inexcitable in 0.11 M diethanol-dimethyl-ammonium chloride.

1, absence of conducted response in the peripheral segment of the nerve; *2*, time line (5 cy); *3* to *20*, restoration by n-amyl-triethyl-ammonium ions; *21* to *24*, restoration by sodium. The sweep speed for record *21* was the same as for record *37* of figure 30.

Note that the fibers of the Et_1 elevation were the first to recover the ability to conduct impulses (record *4*). The Et_1 and Et_2 elevations appear in records *5* to *8*. In records *9* to *12* there appears a third elevation superposed upon descending phase of the Et_2 elevation. In record *17* the spike is not fractionated, but repetitive stimulation prevented the response of the slowest fibers. During the development of the actions of the restoring ions the speed of conduction of the Et_1 fibers decreased relatively more than that of the other fibers so that the Et_1 elevation became a part of the unfractionated spike of record *17*. After restoration by sodium the Et_1 group became distinct during repetitive stimulation (records *22* to *24*). By means of determinations of threshold it was ascertained that the Et_1 elevation was produced by the fibers of the B group.

change in the Et fibers. Treatment of the nerve with Ringer's solution failed to bring about an enhancement of the recovery of fibers of slow conduction (cf. records *21*, *22*, *24*). In contrast with this result, record *23* shows that sodium brought about a recovery of fibers of fast conduction; at the time when record *23* was obtained the number of conducting A fibers still was small, but it was observed to increase during the following hour, until the observations were discontinued.

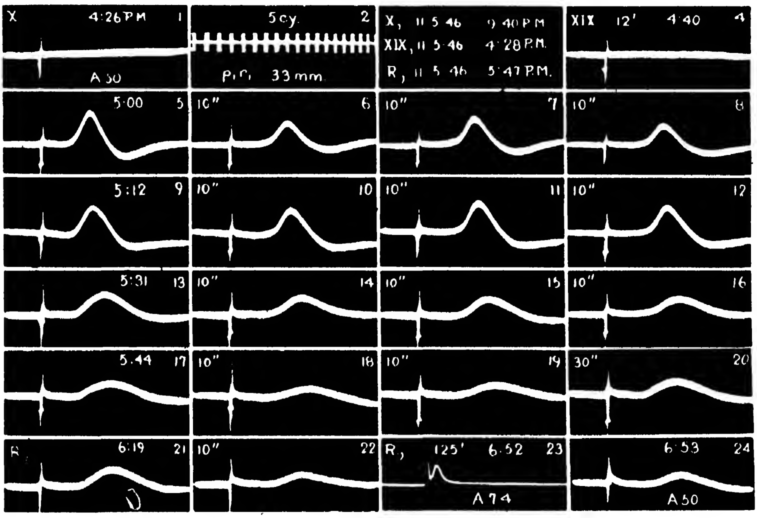


FIG. 32. Restoration of the excitability of the peripheral segment of a nerve rendered inexcitable in a 0.11 M solution of diethanol-dimethyl-ammonium ions.

1, absence of conducted response in the peripheral segment; *2*, time line; *4* to *22*, restoration by n-hexyl-triethyl-ammonium ions; *23*, *24*, restoration by sodium. Note that sodium restored the excitability of A fibers (*23*), but did not improve the state of fibers of slow conduction (*24*).

b. Ions listed in column b of figure 2. The two quaternary ammonium ions with a phenyl group that have been used, have proved to be able to restore the excitability of Et fibers, but as is shown by figure 16 ion XXI is less effective than ion XX. Since the 4th group of ion XXI is the carbon skeleton of adrenaline it seemed advisable to demonstrate the difference between the two ions in a more direct manner.

In the experiment illustrated by figure 33 the observations were begun after the nerve had been 20 hours in a 0.11 M solution of ion X, a length of time that was expected to be sufficient to prevent recovery of excitability except by the action of very effective quaternary ammonium ions. Accordingly it

was found that, even after they had acted upon the nerve for almost one hour, β-phenylethyl-triethyl-ammonium ions were unable to restore the ability to conduct to any nerve fiber (records *2* to *5*). In sharp contrast with this result, it was later observed that phenyl-triethyl-ammonium ions were able to restore probably all the fibers of class Et. The nerve was placed in contact with a 0.11 M solution of phenyl-triethyl-ammonium chloride at 10:50 P.M. and 12 minutes

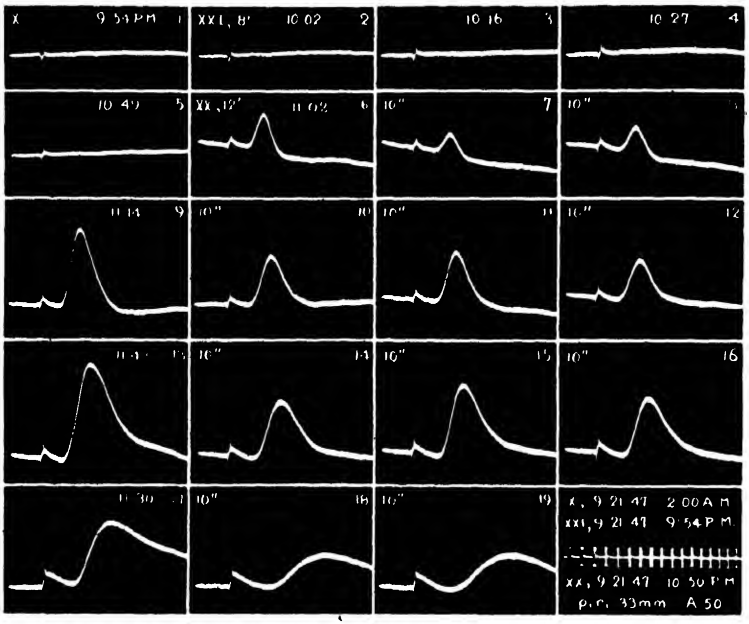


FIG. 33. Restoration of the excitability of the peripheral segment of a nerve rendered inexcitable in 0.11 M diethanol-dimethyl-ammonium chloride.

1, absence of conducted response in the peripheral segment of the nerve; *2* to *5*, lack of restoration by β-phenylethyl-triethyl-ammonium ions; *6* to *19*, restoration by phenyl-triethyl-ammonium ions.

later it was found that a large number of Et fibers had become able to conduct impulses (record *6*) and that many of the restored fibers were able to follow stimulation at 10-second intervals (record *6* to *8*). The restored spike rapidly increased in size (record *9*) and at the time when record *13* was obtained probably all the Et fibers had become able to conduct impulses. Thereafter, both the size of the conducted response and the speed of conduction decreased (records *17* to *19*) for reasons that will be discussed presently.

As already mentioned in section 4,b phenyl-triethyl-ammonium ions are even more effective than tetraethyl-ammonium ions in effecting the restoration

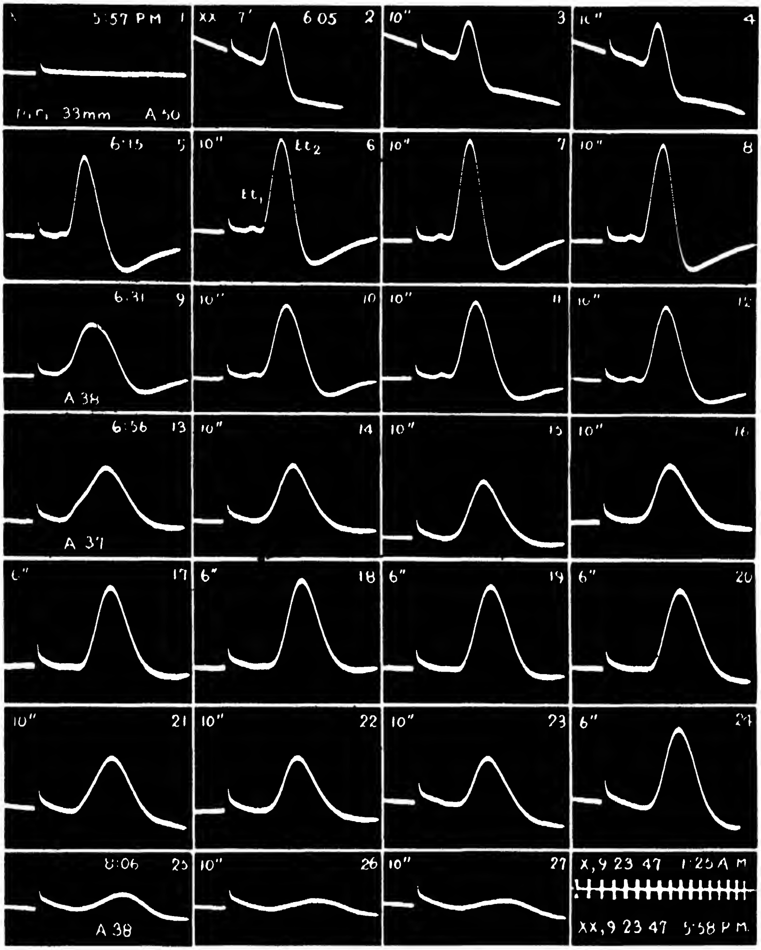


FIG. 34. Restoration of the excitability of the peripheral segment of a nerve rendered inexcitable in 0.11 M diethanol-dimethyl-ammonium chloride.

1, absence of conducted response in the peripheral segment; *2* to *27*, restoration by phenyl-triethyl-ammonium ions.

of Et fibers of nerves deprived of sodium. The action of phenyl-triethyl-ammonium, however, is only temporary, since the response begins to decrease in size shortly after the restoration has become complete and within a relatively short period of time the Et fibers become inexcitable, even though sodium is able to effect again restoration of excitability. The action of tetraethyl-ammonium is more durable, since after their restoration the Et fibers remain excitable at least for many hours.

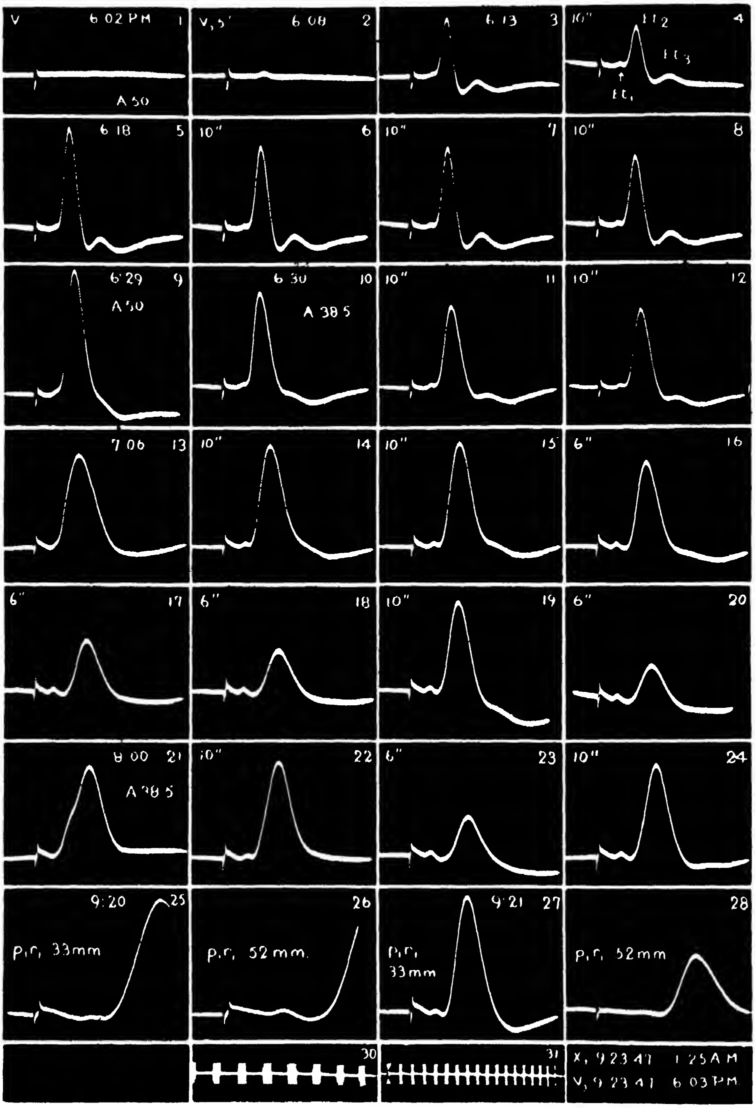


FIG. 35. Restoration of the excitability of the peripheral segment of a nerve rendered inexcitable in 0.11 M diethanol-dimethyl-ammonium chloride.

1, absence of conducted response in the peripheral segment; *2* to *28*, restoration by tetraethyl-ammonium ions. Time line *30* applies to records *25*, *26*; time line *31*, to all the other records. The Roman numeral on the upper left corner of record *1* should be X.

The difference between the actions of ions V and XX was analyzed in detail in the experiment illustrated by figures 34 and 35, which was done with the use of paired nerves. At the time when the observations recorded in figures 34 and 35 were begun the peripheral segments of the nerves had been in a 0.11 M solution of ion X for 16 hours. The nerve used to obtain the records of figure 34 was treated with phenyl-triethyl-ammonium ions, and that used to obtain the records of figure 35 with tetraethyl-ammonium ions.

The initial stages of the recovery are illustrated by records *1* to *8* of figures 34 and 35; as can readily be noted phenyl-triethyl-ammonium initiated the recovery more rapidly than tetraethyl-ammonium, but soon after the recovery by phenyl-triethyl-ammonium had become complete (fig. 34, *5* to *8*) the response began to decrease (fig. 34, *9* to *27*), while in the case of the nerve restored by tetraethyl-ammonium (fig. 35) the response increased throughout the experiment.

A clue to the understanding of the situation was given by the effects of repetitive stimulation of the nerves. In the case of the nerve treated with phenyl-triethyl-ammonium repetitive stimulation at 10-second intervals resulted in an increase of the spike (fig. 34, *5* to *8* and *9* to *12*), while in the case of the nerve treated with tetraethyl-ammonium repetitive stimulation decreased the spike height (fig. 35, *5* to *8; 10* to *12*). The difference between the behaviors of the two nerves became particularly impressive in a later stage of the experiment, when the response of the nerve treated with phenyl-triethyl-ammonium already had undergone an important decrease.

Records *13* to *24* of figure 34 show that when the nerve was stimulated at 10-second intervals the spike height remained low (records *13* to *16* and *21* to *23*), while when the stimulation was applied at 6-second intervals the spike increased to reach practically the height that it had displayed previously (record *5;* note the difference in the amplifications used). In the case of the nerve treated with tetraethyl-ammonium stimulation at 10-second intervals produced a slight increase in the spike height (fig. 35, *13* to *15; 21*, *22*) instead of the decrease that it had previously produced (fig. 35, *5* to *8*), while stimulation at 6-second intervals caused a marked decrease in the spike height (fig. 35, *16* to *20*, *23*).

On the basis of existing knowledge on the nature of the process of recovery after conduction ('47, sections XIV.6 and XV.8) the interpretation of these observations is not difficult. The difference between the two nerves was that which should be expected to exist between two nerves in which the L fraction of the membrane potential has different values; both nerves acquired large L fractions during the restoration of the excitability, but in the nerve treated with phenyl-triethyl-ammonium the L fraction was greater than in the nerve treated with tetraethyl-ammonium.

In all cases, i.e., also when the spike height increased, the speed of conduction decreased; consequently, during repetitive stimulation the nerves were in a state of cathodal depression (relative refractoriness) referable to incomplete recovery of the Q fraction of the membrane potential. With the nerve treated with phenyl-triethyl-ammonium the cathodal depression was mild and consequently the increase in the threshold of stimulation was sufficient to produce only a slight reduction of the speed of conduction; with the nerve treated with tetraethyl-ammonium the cathodal depression was more severe; indeed, in a number of fibers it amounted to absolute refractoriness, with the result that these fibers were unable to conduct impulses at 6-second intervals; hence, the small size of the spike in records *17*, *18*, *20* and *23* of figure 35.

Thus, the difference in behavior of the two nerves was entirely comparable to the difference between a nerve in oxygen and the same nerve in 95% O_2 and 5% CO_2 (cf. '47, section III.6). The action of CO_2 results in an increase in the L fraction of the membrane potential and thereby in an increase in the ability of the nerve to conduct trains of impulses. Similarly, the decrease of the spike (i.e., of the number of conducting fibers) in the case of records *5* to *8* of figure 35 changed into the increase that appears in records *13* to *15* because during the intervening period of time the action of tetraethyl-ammonium ions had resulted in a further increase in the L fraction of the membrane potential and thereby in the functional ability of the nerve.

In regard to the increment in the spike height during repetitive stimulation, the explanation lies at hand. The increment did not represent an increase in the number of conducting fibers, because the stimulation was applied to a segment of nerve that had performed its recovery in Ringer's solution; the increment indicated that the amount by which the L fraction collapsed during the alteration was greater during the second and following spikes of a train than during the first spike (cf. '47, section XIV.6).

Likewise, no difficulty is encountered in explaining the late, progressive decrease of the conducted response of the nerve treated with phenyl-triethyl-ammonium ions. The increment in the L fraction finally raised the stimulation threshold to such an extent that the action current became too small to effect restimulation of the nerve. In other words the increment in the L fraction became large enough to produce an anodal block (cf. '47, section VII.9) comparable to that which is observed with calcium-poisoned nerve (cf. '47, section VII.10). As will be shown in section 8, phenyl-triethyl-ammonium causes a progressive depolarization of the nerve fibers; since at the same time it causes an increment in the L fraction, it is obvious that the Q fraction of the membrane potential undergoes an important decrease. Thus, the state of the nerve is comparable to the state of a veratrine-poisoned nerve, that has repolarized itself in the presence of 5% CO_2 (cf. '47, section II.7); nerves in this state are very susceptible to anodal block.

The extent by which phenyl-triethyl-ammonium ions increase the L fraction of the membrane potential of A fibers is illustrated by the records reproduced in figure 36 that were obtained a few minutes after records *17* to *19* of figure 33. A comparison of figure 36 with figure 47 (*1* to *8*) readily shows that, as measured by the height of the slow catelectrotonus, the L fraction of the membrane potential of the nerve restored by phenyl-triethyl-ammonium ions acting for three hours, was as great as the L fraction of a nerve that had been in the presence of tetraethyl-ammonium ions for 16½ hours. On the other hand, a comparison of figure 36 with figures 4, 8, 17 and 24 shows that the restoration of nerve by tetraethyl-ammonium ions never is accompanied by an increase of the L fraction as great as that which was produced by phenyl-triethyl-ammonium ions in the experiment illustrated by figure 36.

A few details of the records reproduced in figure 36 may be singled out for mention. In the first place attention may be called to the fact that the maximal height of the catelectrotonus in record *1* cannot be regarded as a true measure of the magnitude of the L fraction of the membrane potential since the sharp maximum of the potential indicated that an E_3 nerve reaction of considerable intensity had been produced, which prevented the applied current from removing the L fraction entirely. A more accurate measure of the L fraction is the height of the slow catelectrotonus in record *9* since the broad maximum of this record indicates that the nerve reaction was considerably less effective in opposing the effect of the applied current than in the case of record *1*.

Although the catelectrotonus displayed pronounced E_3 maxima in records *1*, *2* and *4*, the overshooting after the end of the polarization (record *4*) was established at a very low rate; in addition, it was so small as to be almost undetectable. The overshooting was also very small in the case of the catelectrotonus produced by the 15 μa current (record *12*). Likewise, the overshootings of the anelectrotonus were quite small (records *8* and *16*). This behavior of the slow electrotonic potential is characteristic of nerves deprived of sodium (cf. '47, section VIII.4b). Restoration of nerve by quaternary ammonium ions never results in a restoration of the intensity of the nerve reaction to the extent which would be necessary for the production of large overshootings after the end of the polarization (cf. below, section 9).

In the case of the electrotonus produced by the 8 μa current the maximum of the catelectrotonus (records *1*, *2*, *4*) was established with greater rapidity than that of the anelectrotonus (records *5*, *6*, *8*) and the difference between the maximal absolute values of catelectrotonus and anelectrotonus was small. This behavior of the electrotonus is characteristic of nerves with large L fraction that are being polarized with relatively small currents (cf. '47, section XI.3). When the current was increased to 15 μa the anodal maximum became sharper (records *13*, *14*, *16*) and the maximal height of the anelectrotonus became considerably greater than that of the catelectrotonus. A behavior like this is also regularly observed with normal nerves with large L fraction.

Another detail worthy of mention is that in records *14* and *19* the maximal height of the anelectrotonus is slightly less than in records *13* and *18*. The explanation of the phenomenon is that the E_3 reaction established during the pulses used to obtain records *13* and *18* had decreased the polarizability of the membrane (cf. section 9).

c. Comment. The variability of the action upon nerve of quaternary ammonium ions with three ethyl groups depending upon the 4th group again places

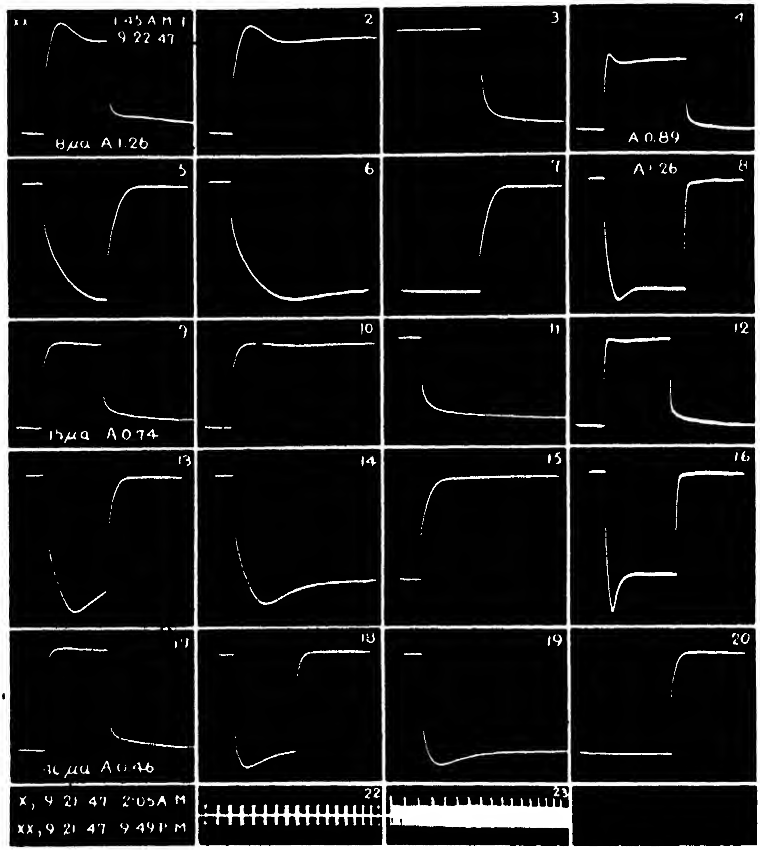


FIG. 36. Electrotonic potentials in a nerve kept in 0.11 M diethanol-dimethyl-ammonium chloride for practically 20 hours, after restoration by phenyl-triethyl-ammonium ions. Time line *23* (5 + 1 cy) applies to records *4*, *8*, *12* and *16*; time line *22* (5 cy) to all the other records. Note that record *4* was obtained at a smaller amplification than the other records of the series *1* to *8*.

in evidence the important fact that substitution of one group in a quaternary ammonium ion may result in important changes in the properties of the ion. Particularly impressive is the result of replacing in tetraethyl-ammonium one ethyl group by one phenyl group. This substitution results in a marked increase of the activity of the ion. If, however, one ethyl group of tetraethyl-ammonium is replaced by the β-phenylethyl group (i.e., by the carbon skeleton

of adrenaline), the activity of the ion is markedly reduced. The general explanation of the phenomenon can be only this. The presence of the phenyl group increases, while the presence of the β-phenylethyl group decreases the affinity of the ion for certain constituents of the nerve fibers. Similarly, it may be concluded from the experimental facts that insofar as the chemical reactions underlying restoration of excitability are concerned, the groups ethyl, n-propyl, n-butyl and n-amyl are almost interchangeable when they enter as the 4th group in a quaternary ammonium ion with three ethyl groups, while the methyl and n-hexyl groups greatly diminish the restoring action of the ion.

The number of compounds with three ethyl groups that have been used in the research presented in this paper is large enough to demonstrate the importance of the rôle that is played by the 4th group; it is, however, far too small for the establishment of general rules.

7. Restoration of Excitability by Quaternary Ammonium Ions without Ethyl Groups

This section deals with experiments done with the use of 0.11 M solutions of tetra-n-propyl-ammonium and tetra-n-butyl-ammonium chloride.

a. Experimental facts. From the three quaternary ammonium ions with 4 identical groups listed in figure 1, I, V and XV, only tetraethyl-ammonium is able to restore the excitability of nerve fibers deprived of sodium. There are, however, two other ions with 4 identical groups that are able to effect restoration of excitability, tetra-n-propyl-ammonium (fig. 2, XXII) and tetra-n-butyl-ammonium (fig. 2, XXIII). These two ions are not as effective as tetraethyl-ammonium, but this circumstance does not diminish the importance of the fact that restoration can be effected by ions that do not contain ethyl groups. Also the fact deserves emphasis that tetra-n-butyl-ammonium ions are more effective than tetra-n-propyl-ammonium ions.

In the experiment illustrated by figure 37 the paired nerves were kept in a 0.11 M solution of ion X for 16½ hours before they were placed in contact with the restoring solutions. As has been routinely done during the research presented in this paper, the observations on the restoration of the peripheral segment of the nerve were not begun until after the central segment had performed a successful recovery in Ringer's solution.

The restoration by tetra-n-butyl-ammonium ions is illustrated by records *1* to *16* of figure 37. The recovery of excitability began approximately as fast as if tetraethyl-ammonium ions had been used. It is true that no nerve fiber had regained its ability to conduct after 7 minutes (record *3*), but a significant number of fibers regained their ability to conduct in 14 minutes. The restored response rapidly increased in size, to become maximal in 19 minutes. As is shown by records *5* to *8*, the restored fibers were able to conduct impulses at 10-second intervals with but little fatigue. Soon thereafter, the restored re-

sponse began to decrease (records *9* to *12; 13* to *15*), and 55 minutes after the restoring solution had come in contact with the nerve the Et fibers were again

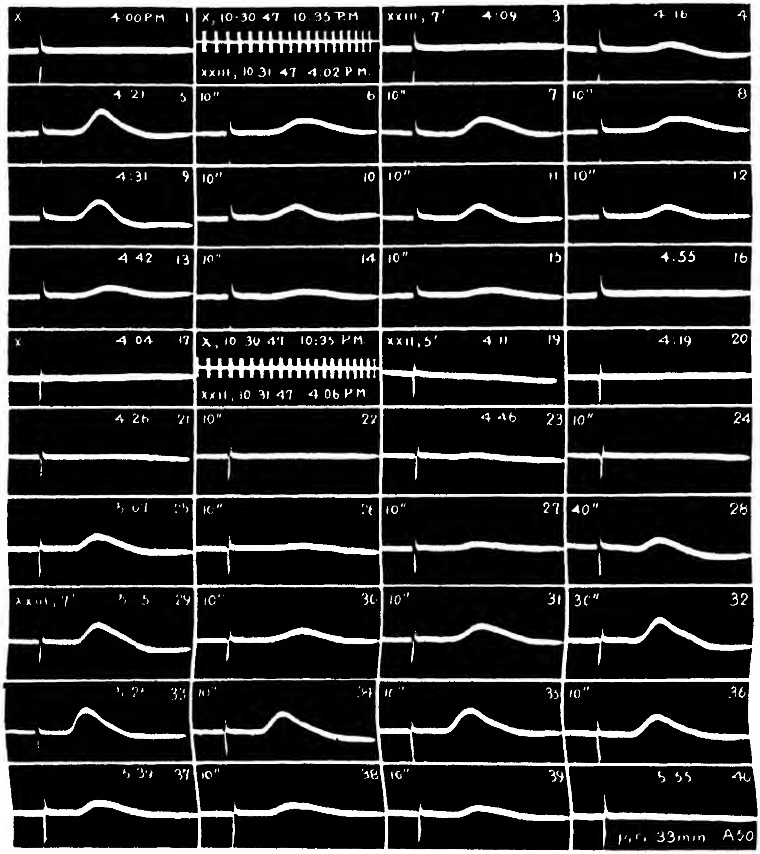


Fig. 37. Restoration of the excitability of the peripheral segments of nerves rendered inexcitable in 0.11 M diethanol-dimethyl-ammonium chloride.

1, absence of conducted response in the peripheral segment; *3* to *16*, restoration by tetra-n-butyl-ammonium ions.

17, absence of conducted response in the peripheral segment; *22* to *28*, restoration by tetra-n-propyl-ammonium ions; *29* to *40*, effect of tetra-n-butyl-ammonium ions.

inexcitable. In all probability, the explanation of the loss of excitability by the restored fibers is that tetra-n-butyl-ammonium ions are a powerful depolarizing agent (cf. section 8); the restoration was produced during the initial phase of the action of the ions, during which the membrane potential is increased, and the loss of excitability occurred during the following phase of depolarization of the nerve fibers.

The restoring action of tetra-n-propyl-ammonium ions began at a very low rate (fig. 37, *19* to *24*); after one hour the response still was quite small and the restored fibers were very susceptible to fatigue (records *25* to *28*). The nerve was then placed in contact with a 0.11 M solution of tetra-n-butyl-ammonium chloride, whereby a rapid enhancement of the recovery was produced. In 7 minutes the tetra-n-butyl-ammonium ions were able to increase the number of conducting fibers (cf. records *25* and *29*) and markedly diminish their fatigability (records *29* to *32*). The restored response still increased in size during the following 10 minutes (records *33* to *36*); thereafter the response decreased (records *37* to *39*) and soon all the nerve fibers proved to be inexcitable (record *40*).

The differences in the effectiveness of ions V, XXII, and XXIII have also been established by direct comparison of their effects on pairs of nerves. In all cases ion V proved to be more effective than ion XXII or XXIII.

b. Comment. In view of the results presented in this section it is clear that no specific rôle can be attributed to the ethyl groups of quaternary ammonium ions in the proceess of restoration of nerve fibers deprived of sodium. On the other hand, it becomes reasonable to believe that the number of quaternary ammonium ions known to be able to effect restoration will eventually become very large. For example, it is to be expected that a number of quaternary ammonium ions having combinations of ethyl, propyl, butyl, amyl and phenyl groups will be able to effect restoration. At any rate, even if no other ions than those examined in this paper were able to effect restoration, which is indeed unlikely, even then the fact would be there that no less than 15 quaternary ammonium ions can restore more or less completely the excitability of Et fibers deprived of sodium.

That the presence in the ion of certain groups or of certain combinations of groups creates better conditions for the restoration than the presence of other groups is doubtless significant; the variety, however, of the groups present in the restoring ions eliminates the possibility of attributing specific properties to any particular group. The only thing that all the restoring ions have in common is the tetracovalent nitrogen nucleus; therefore, the only general conclusion that may be drawn from the experimental observations is this. Tetracovalent (pentavalent) nitrogen, when certain combinations of groups with two or more carbon atoms are attached to it, forms an ion that has the ability to substitute for sodium and to restore the ability to conduct to fibers of slow conduction, as well as certain properties to fibers of the A group.

8. Effect of Quaternary Ammonium Ions on the Resting Membrane Potential

This section presents the results of measurements of changes in the value of the resting membrane potential produced by 0.11 M solutions of the quaternary ammonium ions listed in figures 1 and 2.

a. Interpretation of demarcation potential curves. Since a detailed discussion of the problems encountered in the interpretation of demarcation potential curves has been presented elsewhere ('47, Chapter I), it will be sufficient to call attention here upon a few details.

The demarcation potential curves reproduced in figures 38 to 40 measure the demarcation potential (A–B) measured between a segment of nerve (A) kept in Ringer's solution and another segment (B) in contact with the test solution. The measured potential difference has been plotted with the sign of the untreated segment (A), so that an ascent of the curve indicates that the membrane potential of the treated segment (B) was undergoing a decrease. For the purpose of comparing the different curves the first point of each curve may be regarded as the reference zero for all later measurements, because the initial A–B reading, i.e., the reading made one minute after the nerve had come in contact with the test solution, always was practically equal to the total sum of the liquid junction potentials in the measuring circuit. This sum was determined a few minutes before mounting the nerve in the moist chamber, by establishing junction between the A and B vessels by means of a strip of filter paper soaked with Ringer's solution.

If the membrane potential of the untreated segment of nerve (A) had remained constant during the experiment, no great error would be committed if in comparing the curves reproduced in figures 38 to 40 the assumption were made that changes in the value of the A–B demarcation potential were proportional to the change in the value of the membrane potential of the treated segment (cf. '47, section I.7). As a matter of fact, however, the membrane potential of frog nerve kept in Ringer's solution in an atmosphere of oxygen does not remain approximately constant for longer than a few hours; thereafter the spontaneous deterioration of the nerve causes a progressive decrease in the value of the membrane potential. Thus, as soon as the spontaneous deterioration of the untreated segment (A) begins to progress at a significant rate the changes in the A–B demarcation become approximately proportional to the difference between (1) the change in the value of the membrane potential of the B segment, which are produced by the action of the test solution and (2) the change (decrease) in the value of the membrane potential of the A segment, which is caused by the spontaneous deterioration of the nerve.

As a rule with nerves kept in air or in oxygen the effect of the spontaneous deterioration begins to become significant some 400–500 minutes after excision. If thereafter the depolarizing action of the test solution still progresses at a high rate the course of the demarcation potential curve will not be modified in an appreciable manner by the deterioration of the A segment; if the effect of the test solution is weak the demarcation potential will display a change in slope, it may even pass through a maximum (cf. fig. 38, *2*; fig. 39, *1*).

b. Ions listed in figure 1. The curves reproduced in figure 38 show that among the ions listed in row 1 of figure 1 there is only one, ion IV, that exerts a strong depolarizing action upon nerve (curves *4*, *5*). With the nerves treated with the solutions of the other 4 ions, the treated segment (B) maintained the total value of its membrane potential at approximately the same value as the segment (A)

kept in Ringer's solution, for the whole duration of the experiments (15–17 hours).

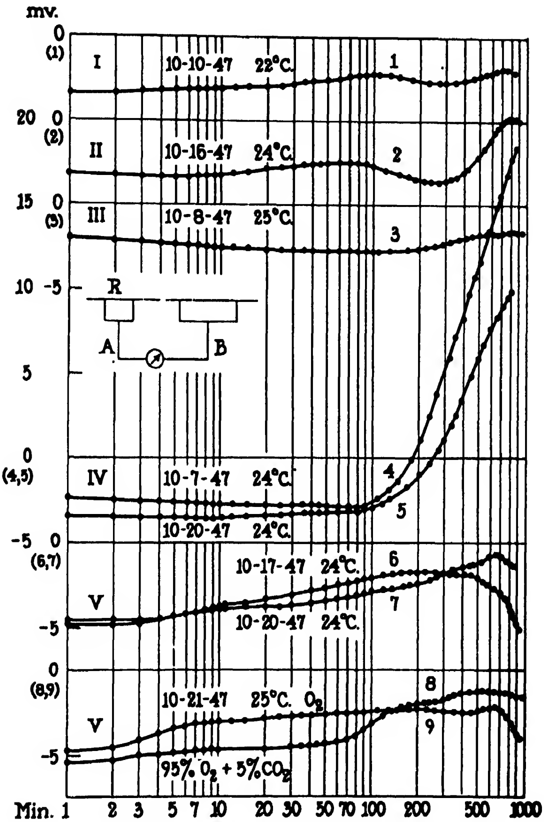


FIG. 38. Demarcation potential curves resulting from the action of 0.11 M solutions of quaternary ammonium ions upon the B segments of the nerves. Except in the case of curve *8* the nerves were kept in an atmosphere of oxygen. The Roman numerals identify the ions used. The demarcation potential has been plotted with the sign of the *A* segment.

No comment need be made on curves *1* and *3* of figure 38, since these curves do not present significant deflections. Curve *2* displays an oscillatory course, but the deflections are so small that they can hardly be regarded as significant, especially because the second-phase of ascent began late and was followed by a well-defined maximum.

The meaning of the late maximum was analyzed experimentally in the case of compound V (tetraethyl-ammonium). According to the remarks made in

the preceding subsection the ultimate descent of the *A–B* curve would indicate that in the untreated segment the membrane potential was undergoing a decrease, referable to spontaneous deterioration of the nerve, faster than that in the treated segment. If this were the case, it should be expected that the terminal, descending segments of curves *6* and *7* would not appear if the nerve were kept in an atmosphere of 95% O_2 and 5% CO_2 because the presence of 5% CO_2 in the atmosphere of the nerve is known markedly to delay the spontaneous deterioration of frog nerve. In point of fact, curve *9* that was obtained with the nerve in an atmosphere of oxygen presents a terminal, descending segment while curve *8* that was obtained with the nerve in an atmosphere of 95% O_2 and 5% CO_2 does not present a late descending phase.

Demarcation potential curves measuring the effects of the 10 other ions listed in figure 1 are presented in figure 39. As can readily be noted, ions IX, X and XII do not produce a significant change in the total value of the membrane potential (curves *4*, *5* and *7*). The effect of choline (curve *6*) also is exceedingly small.[7] The remaining ions, however, exert a definite depolarizing action upon nerve.

If curves *2*, *3*, *6*, *8*, *9* and *10* of figure 39 are examined with some attention, it is found that they belong to two different classes. In the case of curves *2*, *3*, *6* and *8*, shortly after the phase of depolarization began, the value of the demarcation potential increased above the initial *A–B* reading more or less linearly in the logarithm of the time and the effect of the deterioration of the untreated segment did not modify the demarcation potential in an appreciable manner (curves *2*, *3*) or produced only a small change in the slope of the curve (curves *6*, *8*). Thus, although the depolarizing action of ions VII and XIII is less powerful than that of ions VIII and XI, all 4 ions produce a continuously increasing depolarization of the nerve fibers. On the other hand, ions IV, VIII and XI are equally strong depolarizing agents. In view of the demarcation potential curves obtained with the use of these ions (fig. 38, *4*, *5*; fig. 39, *3*, *6*) it cannot be a surprising fact that ions IV, VIII and XI ultimately cause irreversible deterioration of the nerve fibers. The smaller depolarization produced by ions VII and XIII is in agreement with the fact that the effect of these two ions is reversible (cf. section 5,d).

Curves *9* and *10* of figure 39 belong to a different type, since the phase of ascent is irregular and the curves end with nearly horizontal segments. Thus, ions XIV and XV can cause only a limited decrease in the value of the membrane potential, which is in agreement with the facts presented in section 5,b and c.

[7] Curve *1* of figure 39 is essentially identical with the *A–B* curves previously published ('47, fig. VIII.14, n.IV); the *A–B* potential, however, has been plotted here with the sign of the untreated (*A*) segment.

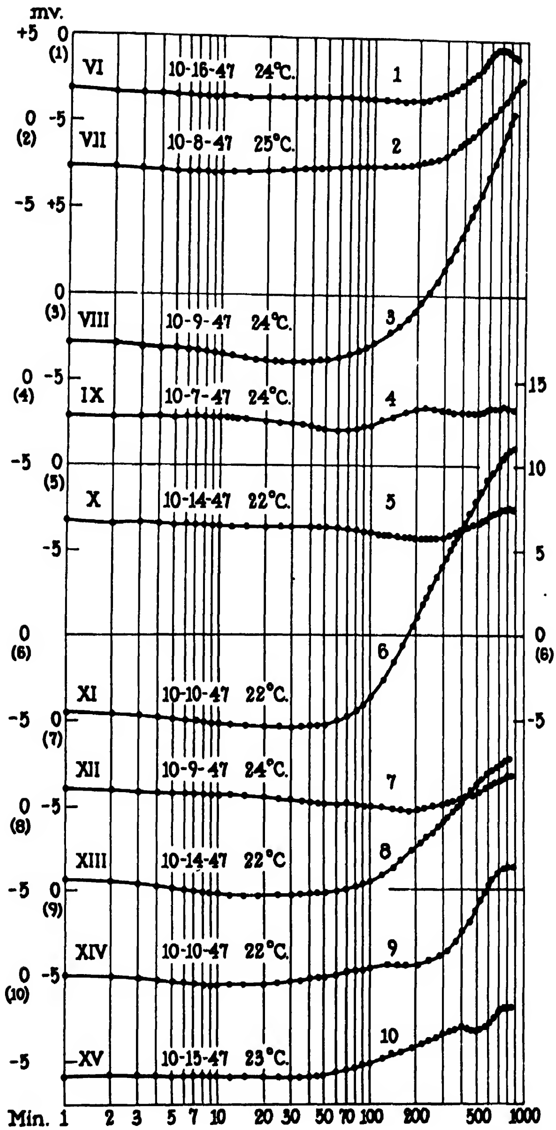


FIG. 39. Demarcation potential curves resulting from the action of 0.11 M solutions of quaternary ammonium ions upon the *B* segments of the nerves. Nerves in oxygen.

c. Ions listed in figure 2. The curves reproduced in figure 40 illustrate the effects of a number of the ions listed in figure 2. It will be noted that all the ions used to obtain the curves of figure 40 exert a depolarizing action upon nerve.

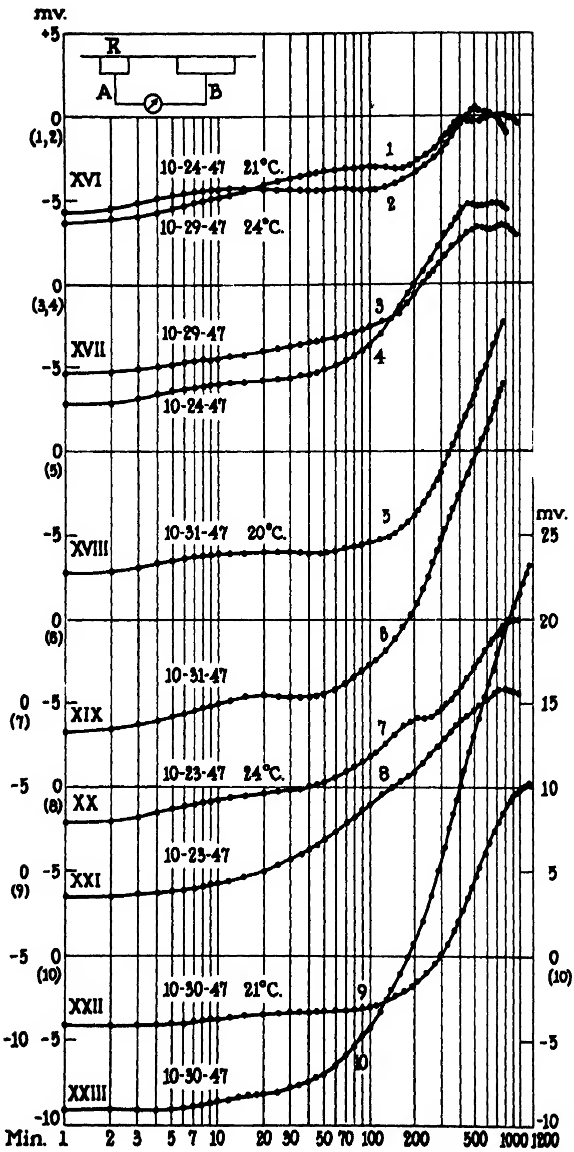


FIG. 40. Demarcation potential curves resulting from the action of 0.11 M solutions of quaternary ammonium ions upon the *B* segments of the nerves. Nerves in oxygen.

A remarkable fact is that in the series of ions with three ethyl groups, XVI to XIX, the depolarizing action increases with the length of the carbon chain of the 4th group, to become very powerful when the chain has 6 carbon atoms

(n-hexyl-triethyl-ammonium, fig. 2, XIX). If the effects of ions IV (fig. 38, *4*, *5*) and V (fig. 38, *6* to *9*) also are taken into account, it appears that in the series of ions with three ethyl groups (fig. 2, column a) tetraethyl-ammonium occupies a unique position, since it is the only ion in the series that exerts no depolarizing action upon the nerve fibers. The discontinuities in the change of properties that result from lengthening the carbon chain from 1 carbon (compound IV) to 6 carbon atoms (compound XIX) certainly deserve emphasis.

Ions XX and XXI also depolarize the nerve fibers. The fact, however, that curves *7* and *8* of figure 40 end with nearly horizontal segments indicates that the depolarization produced by these ions is not very severe.

The three ions with 4 identical groups listed in figure 1 (I, V, XV) do not depolarize nerve (I, V) or only slightly (XV); tetra-n-propyl-ammonium and tetra-n-butyl-ammonium (fig. 2, XXII and XXIII), however, are powerful depolarizing agents. Indeed, among the quaternary ammonium ions that have been used in the research presented here, ion XXIII is the strongest depolarizing agent. Indeed, a 0.11 M solution of ion XXIII depolarizes nerve almost as fast as a 0.11 M solution of K^+ ions.

In one experiment done with the use of compound XXIV it was found that this ion does not alter the membrane potential of nerve in a significant manner. Finally, as was demonstrated elsewhere ('44; '47, section IV.7) acetylcholine (fig. 2, XXV) exerts no depolarizing action of its own upon nerve.

9. *Interpretations*

This section is dedicated to the analysis of the experimental results concerning fibers of fast conduction which have been presented in sections 3 to 8. Use is made in the analysis of information offered in recent publications ('46, '47) on the nature of the electrotonic potential and of the resting membrane potential.

a. Preliminary remarks. The *A–B* demarcation potential of a mixed nerve like a frog sciatic nerve is, of course, referable to all those fibers the properties of which have been modified by the test solution. Even in the case, however, that the test solution should act upon all the fibers of the nerve in the same manner the measured external demarcation potential would be referable in the main to the fibers of the A group. This interpretation can be based upon observations made with the use of nerves in which the fibers of fast conduction had been destroyed by the action of a large excess of calcium (cf. '47, section II.4b); with nerves in such a state the demarcation potential measured against a segment treated with 0.11 M KCl is only a small fraction of the demarcation potential measured with normal nerves. To be sure, no informatioh is available to rule out the possibility that the effect of calcium may have produced an important decrease in the membrane potential of the fibers of slow conduction, but the interpretation can also be based upon the analogy of the situations that prevail during the measurements of the demarcation and of the action potential (cf. '47, sections I.1 and V.4). Since the crest height of the external action potential of the B and C fibers is only a small fraction of the crest height the external action potential

of the A fibers, the external demarcation potential of B and C fibers cannot be expected to be more than a small fraction of the external demarcation potential of the A fibers. The resting membrane potential as well as the membrane action potential of all the nerve fibers probably are of the same order of magnitude; with fibers of small caliber, however, the high longitudinal resistance of the core necessarily makes the external potential smaller than it is in the case of fibers of large caliber. Moreover, it should be expected that longitudinal polarization of the core would reduce the external potential more in the case of fibers of small caliber than in the case of fibers of large caliber.

A similar situation is encountered in the study of the electrotonic potential; as was demonstrated elsewhere (cf. '47, section VI.7), the external electrotonic potential of frog nerve is referable in the main to the fibers of fast conduction. In the discussion that follows, both the *A–B* demarcation potential and the electrotonic potential will be regarded as measuring changes in the properties of the A fibers.

It is also important to note that the *A–B* demarcation potential measures changes in the total value of the resting membrane potential; it yields no information on the relative value of the fractions of the membrane potential. Information of this kind can be obtained only by means of a study of the after-potential and of the electrotonic potential. With inexcitable nerve, study of the electrotonic potential is the only available method of analysis of the fractions of the membrane potential.

At the present state of knowledge it is advisable or rather necessary to limit the scope of the analysis by using the simplifying assumption that the membrane potential has only two fractions, Q and L (cf. '46; '47, Chapter XIV) and that the electrotonic potential has only two components, the fast and the slow component (cf. '47, section VI.3).

b. Analysis of the depolarization of nerve by quaternary ammonium ions. In analyzing the curves reproduced in figures 38 and 39, the first fact that comes to one's attention is the late onset of the phase of depolarization of the nerves treated with solutions of ions IV (fig. 38, *4*, *5*), VIII (fig. 39, *3*) and XI (fig. 39, *6*). As a matter of fact, in the case of curves *3* and *6* of figure 39, the phase of depolarization was preceded by a phase of slight hyperpolarization, and there can be hardly any doubt that the initial, practically horizontal segments of curves *4*, *5* of figure 38 correspond to the phases of hyperpolarization of curves *3* and *6* of figure 39.

The significance of the initial phases of the demarcation potential curves is not difficult to ascertain. The records reproduced in figure 7 demonstrate that the action of ion IV upon the nerve fibers resulted in an increase in the L fraction of the membrane potential. Similar observations have been made with the use of ion VIII. Therefore, the depolarization of nerve by ions IV and VIII, and probably also the depolarization by ion XI begins with an increase in the L fraction of the membrane potential and a decrease in the Q fraction. The L/Q ratio remains high during the initial part of the phase of depolarization, but oscillographic analysis of the electrotonic potential has shown that after

the $A-B$ potential has increased by a few millivolts above the initial $A-B$ reading, the L fraction becomes negligible.

It is important to note that this behavior of nerves that are being depolarized by quaternary ammonium ions has no specific traits, since similar observations have been made with nerve poisoned with a large excess of Ca^{++} ions, with iodoacetamide or with cyanide (cf. '47, section XV.4). The variety of the agents that produce similar results leaves no alternative but to conclude that the behavior of the nerve is not determined by the nature of the depolarizing agent; it is determined primarily by the nature of the mechanism that maintains the resting membrane potential. As soon as the depolarizing agent has caused a decrease in the value of the Q fraction, the metabolic mechanisms are set into activity to compensate for the loss, i.e., the decrease in the membrane potential elicits a nerve reaction, similar to that which is elicited during the flow of an applied cathodal current, and since an increment in the L fraction is the preliminary step in the creation of the membrane potential, the L fraction is increased even to the extent of overcompensating the decrease in the total value of the membrane potential. The late phase of rapid decrease of the membrane potential, indicates that the metabolic mechanisms have become unable to counteract the depolarizing action of the test substance.

It is also important to note that the general course of the demarcation potential curves resulting from the action of quaternary ammonium ions has no specific traits, since the demarcation potential curves resemble not only the curves produced by the agents mentioned above, but also the curves that are obtained with the use of certain monovalent ions (NH_4^+, Cs^+, Li^+; Gallego and Lorente de Nó, '47). The similarity of the temporal course of the depolarizations produced by agents of great diversity clearly indicates that although the processes initiating the depolarization may be different in detail, the process underlying the depolarization is essentially the same in all cases. Perhaps the best procedure to reach a general understanding of the situation is to consider the results of depriving the nerve of oxygen (cf. '47, section I.7 and Chapters II and XIII). In all probability, the anoxic depolarization represents the consumption of a store of oxidized substances, that are necessary for the maintenance of the membrane potential and that, in the absence of oxygen, cannot be synthesized by the metabolic mechanisms.[8] By analogy the following assumption may be made. The depolarization of nerve by quaternary ammonium ions is referable to interference with metabolic synthesis of substances that are required for the maintenance of the resting membrane potential. The depolarizing ions enter into chemical combination with constituents of the nerve fibers and thereby alter the normal course of metabolic reactions; in other words, the

[8] Nevertheless, to a large extent an applied anodal current may substitute for oxidative metabolism (cf. '47, Chapter XIII).

quaternary ammonium ions of the depolarizing class play the rôle of metabolic inhibitors.

The curves reproduced in figure 40 seem to belong to a type different from that of curves *3* and *6* of figure 39 since in all of them the phase of depolarization begins immediately after establishment of contact of the nerve with the test solutions. As a matter of fact there is no essential difference between the initiation of the depolarization by ions XVI to XXIII and by ions IV or VIII. According to curve *7* of figure 40 phenyl-triethyl-ammonium ions rapidly initiate a depolarization of the nerve fibers; nevertheless, as was described in section 6,b, phenyl-triethyl-ammonium ions produce a large increase in the L fraction of the membrane potential. Consequently, the depolarization is referable to a decrease in the Q fraction, which is not fully compensated by the increase in the L fraction. Similar observations have been made with ions XVI, XVII, XVIII and XXII.

In addition, a systematic analysis has been made of the effects of tetra-n-butyl-ammonium ions (fig. 2, XXIII), at concentrations ranging from 0.011 to 0.11 M, upon the membrane potential. At all the concentrations used these ions produce a depolarization of the nerve fibers, but at the smaller concentrations, and in one case at the 0.11 M concentration, tetra-n-butyl-ammonium ions produced an initial phase of hyperpolarization. Remarkably enough, the family of demarcation potential curves obtained with the use of tetra-n-butyl-ammonium ions closely resemble the family that was obtained with the use of Li^+ ions at the same concentrations (Gallego and Lorente de Nó, '47, fig. 5).

c. Inert and restoring ions. Nerves kept in 0.11 M solutions of the inert ions I, II, X, maintain their membrane potential at the same level as nerves kept in Ringer's solution; nevertheless, the properties of the former are very different from the properties of the latter nerves. This phenomenon will be analyzed now in detail.

Before starting the analysis it will be useful to repeat that the changes undergone by nerves kept in a 0.11 M solution of one of the inert ions are referable solely to the lack of sodium ions in the external medium of the nerve fibers, since essentially the same changes are observed with nerves kept in a solution of saccharose.

There are two significant details in the electrotonic potential of nerves that have become inexcitable in solutions of inert ions, (1) the catelectrotonus does does not display a slow component, which shows that the L fraction of the membrane potential has a negligible value and (2) the slow anelectrotonus has different temporal courses in the case of small and in the case of large applied currents. The second detail appears with particular clarity in figure 41. The slow anelectrotonus produced by the 8 μa current (records *2* to *4*) did not display a maximum during the polarization; instead, it increased continuously at a small rate during the flow of the applied current to approach a constant

value toward the end of the 10-second pulse used to obtain records *3* and *4*.[9] With the 15 μa current the anelectrotonus (records *6* to *8*) also began to be established at a small rate; after the applied current had flowed for approximately 200 msec the tracing of the anelectrotonus displayed a sharp change in

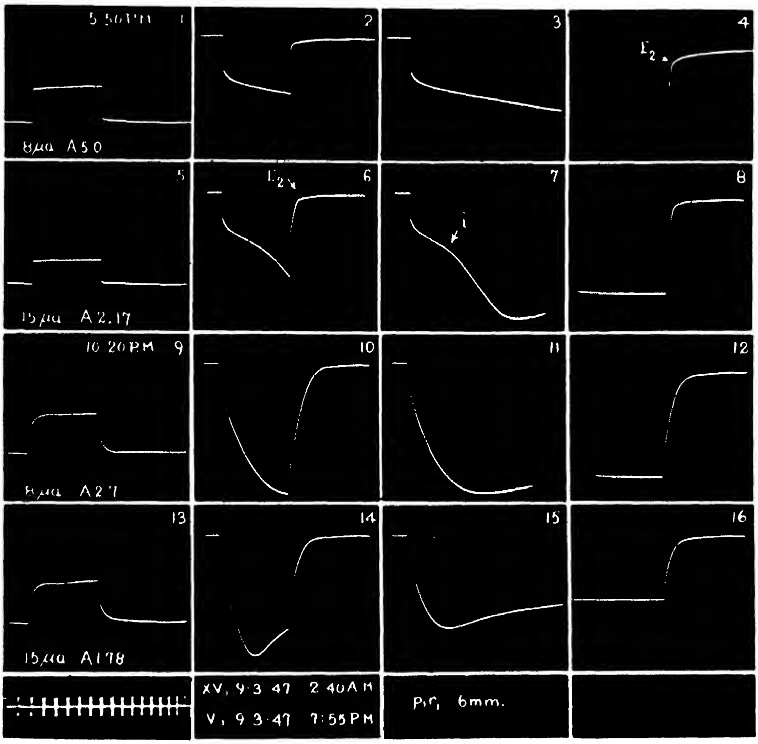


FIG. 41. Electrotonic potentials in a nerve kept in 0.11 M tetraethanol-ammonium chloride, before (*1* to *8*) and after restoration by tetraethyl-ammonium ions (*9* to *16*).

slope (cf. arrow *i* on record *7*); then, after a period of rapid increase in height, the anelectrotonus passed through a pronounced maximum.

A behavior of the slow anelectrotonus such as is illustrated by records *2* to *4* and *6* to *8* of figure 41 is never observed with normal nerve; to cause its appearance it is necessary to submit the nerve to the effect of depolarizing agents or to

[9] By themselves records *3* and *4* are not sufficient to substantiate this statement, since approximately 6.5 seconds elapsed between the end of the sweep of record *3* and the start of the sweep of record *4*. The statement is based upon visual observation of the milammeter that measures the plate current in the last stage of the amplifier as well as on observations of oscillograph's tracings at lower sweep speed and smaller amplification.

a lowering of the temperature. An example of the phenomenon was discussed in detail elsewhere ('47, section XIII.1d). The increase in the slope of the tracing of the slow anelectrotonus is the first sign of the restoration of the properties of the membrane by the anodal current. It indicates that the increment in the L fraction produced by the current has increased the polarizability of the membrane and consequently the rate of the further growth of the slow anelectrotonus. The second sign of the restoration is the appearance of a maximum in the anelectrotonus; the fact that the potential passes through a maximum proves that the restoration of the membrane has gone so far that an E_3 reaction is produced, which reduces the value of the anelectrotonus (see below). This interpretation is in agreement with the observations made after restoration of the nerve by tetraethyl-ammonium ions. The restoration resulted in a large increase in the L fraction of the membrane potential and consequently in the appearance of large slow components in the catelectrotonus (fig. 41, *9*, *13*); after restoration of the nerve the slow anelectrotonus did not display inflection points during its establishment; moreover, it passed through maxima as well in the case of the 8 μa current (records *10* to *12*) as in that of the 15 μa current (records *14* to *16*).

The nerve used to obtain records *1* to *8* of figure 41 probably had undergone a slight depolarization (cf. fig. 39, *10*), but the total value of the membrane potential of the nerve used to obtain the records of figure 42 undoubtedly was equal to that of a nerve kept in Ringer's solution for the same length of time (fig. 39, *5*); nevertheless, the records of the establishment of the anelectrotonus (*2*, *3*, *6*, *7*) present inflection points (marked by arrows) that undoubtedly had the same significance as the inflection points of records *6* and *7* of figure 41. Similar observations can be made in other figures.

The state of the membrane of the nerve fibers at the time when records *1* to *8* of figure 7 were obtained was quite similar to the state which is defined by records *1* to *8* of figure 41. The 8 μa current produced a continuously increasing slow electrotonus (fig. 7, *2* to *4*), while the slow anelectrotonus created by the 15 μa current after it had displayed the inflection point which is marked by the arrow (*i*) passed through a maximum (records *7*, *8*). When the current was increased to 40 μa it was found that both the inflection point and the maximum appeared earlier during the polarization. A result like this should be expected, since the restoring ability of the 40 μa anodal current is, of course, greater than that of the 15 μa current. As a matter of fact, in the case of record *10* (fig. 7) the restoration of the polarizability of the membrane occurred so rapidly that the inflection point is hardly detectable in the phase of increase of the anelectrotonus; the inflection point is clearly visible in record *11* because the E_3 reaction produced during the pulse of record *10* had decreased the polarizability of the membrane (see below).

After methyl-triethyl-ammonium ions had produced a partial restoration of

the nerve, the restoring effect of the anodal current became more pronounced (fig. 7, *14* to *16* and *18* to *20*); after tetraethyl-ammonium ions had further improved the state of the nerve, the inflection points in the tracings of the anelectrotonus produced by 8 and 15 μa currents became hardly detectable (fig. 8, *2* to *4*, *6* to *8*) and no inflection point was observed in the anelectrotonus produced by the 40 μa current (fig. 8, *10* to *12*); finally, after the action of Na^+ ions the slow anelectrotonus was established in normal fashion (fig. 8, *14* to *16*, *18* to *20*, *22* to *24*).

If the nerves are kept in the 0.11 M solution of an inert ion for several hours after all the fibers of the nerve have lost their ability to conduct impulses the polarizability of the membrane decreases so far that even the 15 μa anodal current is unable to restore the membrane to the extent that the slow anelectrotonus displays a maximum during the polarization. This was the case in the experiments illustrated by figures 4 and 9. In the experiment of figure 4 the loss of polarizability of the membrane was less important than in the experiment of fig. 9. Accordingly, records *9* to *12* of figure 4 show that tetraethyl-ammonium ions were able to improve markedly the state of the nerve (cf. section 3,a), while in the experiment of figure 9 the restoration, first by dimethyl-diethyl-ammonium ions (records *9* to *16*) and then by tetraethyl-ammonium ions (records *17* to *24*) was quite limited. Proof of the restoring action exerted by dimethyl-diethyl-ammonium ions was the appearance of an inflection point in the slow anelectrotonus produced by the 15 μa current (record *15*); after the action of tetraethyl-ammonium ions an inflection point appeared already when the nerve was polarized with the 8 μa current (record *19*) and when the 15 μa current was used the inflection point was followed by a maximum of the anelectrotonus (record 23). Thus, there can be no doubt that despite the advanced stage of the effect of the lack of Na^+ ions, the quaternary ammonium ions had induced changes in the membrane, which enabled the anodal current to exert its restoring effect to a higher degree than at the start of the observations (records *1* to *8*).

In an incidental fashion, so as to avoid an excessive lengthening of the discussion, it may be mentioned here that the state of the nerve fibers defined by records *1* to *8* of figure 9 represents one of the most advanced, reversible changes in the properties of the nerve that have been observed in experiments done with the use of solutions of the inert, quaternary ammonium ions or of saccharose. Although the total value of the membrane potential must have been practically the same as in a nerve kept in Ringer's solution (cf. fig. 38, *2*), the lack of Na^+ ions had resulted in changes in the electrotonic potentials such as are observed with nerves in an advanced state of depolarization or at temperatures low enough to produce inexcitability of all the nerve fibers. The anodal current was able to produce only a very small slow anelectrotonus, and what is even more significant, the fast electrotonus was greater at the end of the applied pulses (records *4*, *8*) than at the start of the polarization (records *3*, *7*). On the basis of the results of other similar experiments it may be stated that if the nerve

had been kept in the 0.11 M solution of ethyl-trimethyl-ammonium ions for a few additional hours those observations would have been reproduced with this nerve, which were reported elsewhere ('47, figs. VIII.5 to VIII.9) for severely depolarized nerves or nerves at very low temperatures, on the dependence of the fast elecrotonic deflections upon the effect of long lasting, applied polarization. Anodal polarization would have increased and cathodal polarization would have decreased the fast electrotonic deflections.

An inequality of the two fast electrotonic deflections, such as is illustrated by records *3*, *4* and *7*, *8* of figure 9 indicates that the chemical mechanisms in the membrane can perform only a small part of their normal rôle, or otherwise stated that the membrane is acquiring the properties of inert electrochemical systems (cf. '47, Chapter VIII). When nerves in this state are submitted to the effect of suitable restoring agents, one of the earliest signs of restoration is that the two fast deflections, that at the start and that at the end of an anodal pulse, become approximately equal; this phenomenon indicates that certain regulatory mechanisms in the membrane have again become effective (cf. '47, sections VIII.3 and XV.5c). In the experiment that is now under consideration the degree of restoration effected by dimethyl-diethyl-ammonium ions was not sufficient to produce equality of the two fast deflections, but these deflections became equal after the nerve had been submitted to the effect of tetraethyl-ammonium ions (records *19*, *20*; *23*, *24*).

It will be useful to restate the main points established thus far. In a nerve deprived of sodium in a solution of one of the inert, quaternary ammonium ions or of saccharose the membrane potential maintains its total value at the same level as in nerves kept in Ringer's solution for the same length of time; an important change, however, takes place in the constitution of the potential and in the properties of the membrane. The L fraction of the membrane potential disappears or rather becomes so small as to be undetectable[10] and the polariz-

[10] In nerves that are being kept in Ringer's solution in an atmosphere of air or oxygen the L fraction may have a negligible value if the nerve is in or on the verge of the rhythmic state (cf. '47, section III.8); nevertheless, with nerves in the rhythmic state *the polarizability of the membrane by the anodal current is high, so that the anodal current can produce slow anelectrotonus, i.e., an increment in the L fraction, in the normal fashion.* On the other hand, introduction of 5% CO_2 into the atmosphere of the nerve rapidly results in a large increase in the L fraction, whereby the nerve passes into the resting state. If the nerve kept in Ringer's solution has been able to make the transition into the pseudoresting state, the L fraction will have a large value. Whether or not a nerve kept in Ringer's solution in air or oxygen will be able to make the transition into the pseudoresting state, i.e., to compensate for the absence of CO_2 in its atmosphere, undoubtedly depends upon metabolic conditions in the nerve itself, which of course depend upon the metabolic conditions in the body of the frog from which the nerve has been taken (cf. '47, section XV.4, p. 314); indeed, according to observations made in collaboration with Dr. C. L. Cazzullo, the addition of a small amount of sodium ascorbate to Ringer's solution greatly favors the transition into the pseudoresting state of nerves kept in oxygen. To be more specific, a nerve kept in

ability of the boundary (l–m) at which this fraction is normally maintained becomes so low that anodal currents of moderate magnitude are able to create slow anelectrotonus, i.e., to increase the L fraction of the membrane potential only by very small amounts; in addition, the temporal course of the slow anelectrotonus is abnormal, since the anelectrotonus does not display a maximum during the flow of the applied current. If the absence of Na^+ ions has not been prolonged beyond a certain limit, larger nodal currents are able to increase the polarizability of the membrane and after this restoring effect of the current has been produced, the further flow of the current creates slow anelectrotonus having a temporal course that resembles the normal pattern.

The changes produced by the lack of Na^+ ions are reversed by quaternary ammonium ions of the restoring type, insofar as these ions increase the L fraction of the membrane potential and the polarizability of the boundary (l–m) at which the L fraction is maintained, with the result that even small anodal currents become able to produce slow anelectrotonus that has large magnitude and passes through a maximum during the flow of the applied current.

A further step in the analysis can be undertaken by studying the effects of quaternary ammonium ions on the nerve reaction of nerves deprived of sodium. The study will also contribute to the understanding of the nerve reaction itself.

As was demonstrated elsewhere ('47, section VIII.4b) the slow electrotonic potential of nerves deprived of sodium does not reverse its sign after the end of the polarization, even in those cases in which the potential displays a maximum during the flow of the applied current. The lack of an overshooting of the electrotonic potential after the end of the polarization indicates that during the flow of the applied current the nerve reaction has been established in a defective manner. Indeed, since the electrotonic potential may pass through a maximum in nerves that have become inert systems (cf. '47, section VIII.4c) the lack of the overshooting might be taken as a proof that the nerve reaction has not been produced. According to the evidence that is now available the lack of the overshooting indicates only that the nerve reaction has been established in a defective manner.

---

oxygen in Ringer's solution containing sodium ascorbate at the concentration 0.005 M behaves much in the manner of the companion nerve kept in Ringer's solution in the presence of 5% CO_2.

The essential characteristic of nerves kept in solutions of inert, quaternary ammonium ions or in Ringer's solution at a low temperature, is not only that the L fraction of the membrane potential has a negligible value, it is chiefly that the applied anodal current cannot increase the L fraction except by a moderate amount and in an abnormal fashion. As will be explained later in the text, the low polarizability of the membrane by the anodal current is referable to the failure of certain chemical reactions of the metabolic chain to take place at a sufficient rate and to a sufficient extent (cf. also, '47, sections XIII.1d, XV.4 and XV.5c).

Records *1, 2; 9, 10*, and *11, 12* of figure 42 were obtained with the use of 8 μa applied currents; *1* and *2* with the unconditioned nerve, *9* and *10*, respectively 10 and 20 seconds after the 10-second period of anodal polarization with a 15 μa current used for records *7* and *8*, and *11* and *12*, repectively 10 and 20 seconds after a 10-second period of anodal polarization with a 40 μa current. It will be noted that the slow component is much smaller in record *10* than in record *2*, and that in record *12* the slow component is almost undetectable. After the nerve had been allowed to rest for 10 minutes the anelectrotonus again displayed a slow component *identical with that of record 2. Thus, there is the fact* that, while a brief flow of anodal current *increased* the polarizability of the membrane (cf. inflection points *i* in *records 2, 3, 6, 7*), a long lasting flow resulted in a great loss of polarizability from which the nerve fibers recovered at an exceedingly low rate.

The loss of polarizability of the membrane or rather of the l-m boundary, which was produced by the anodal current, is not an abnormal phenomenon, since a similar phenomenon can be observed with freshly excised nerves kept in Ringer's solution in an atmosphere of O_2 or of 95% O_2 and 5% CO_2 (cf. '47, section VIII.8, XI.3 and XII.2b). An important difference exists, however, between normal nerve and nerve deprived of sodium.[11] With normal nerve the loss of polarizability occurs together with a decrease in the value of the membrane potential (postanodal overshooting), the loss of polarizability and the depolarization having parallel temporal courses; with nerves deprived of sodium the loss of polarizability occurs in the absence of a measurable change in the value of the membrane potential, since after the end of the polarization the electrotonic potential does not reverse its sign.

That in nerve deprived of sodium the loss of polarizability resulting from anodal polarization is caused by the operation of the nerve reaction is a conclusion to be drawn immediately from the experimental results. Since during the initial part of its flow the anodal current increases the polarizability of the membrane (cf. inflection points *i* in records *2, 3, 6, 7* of fig. 42) it is clear that the applied current cannot be the direct cause of the loss of polarizability; the loss must be the result of a secondary process taking place in the nerve fibers themselves. Moreover, there is a close correspondence between the establishment of the maximum of the anelectrotonus and the creation of the loss of polarizability. If the applied current is interrupted before the anelectrotonus reaches its maximum the loss of polarizability, if it is at all detectable, is only slight. For example, it will be noted in figure 42 that the applied pulse of record

[11] Excellent examples of the loss of polarizability caused by anodal polarization in nerve restored by sodium are records *18, 19* and *22, 23* of figure 24. In some cases, however, the loss of polarizability is difficult to demonstrate with sodium-treated nerves because the recovery from the loss is so rapid that the loss remaining 10 seconds after the end of the applied pulse is almost negligible.

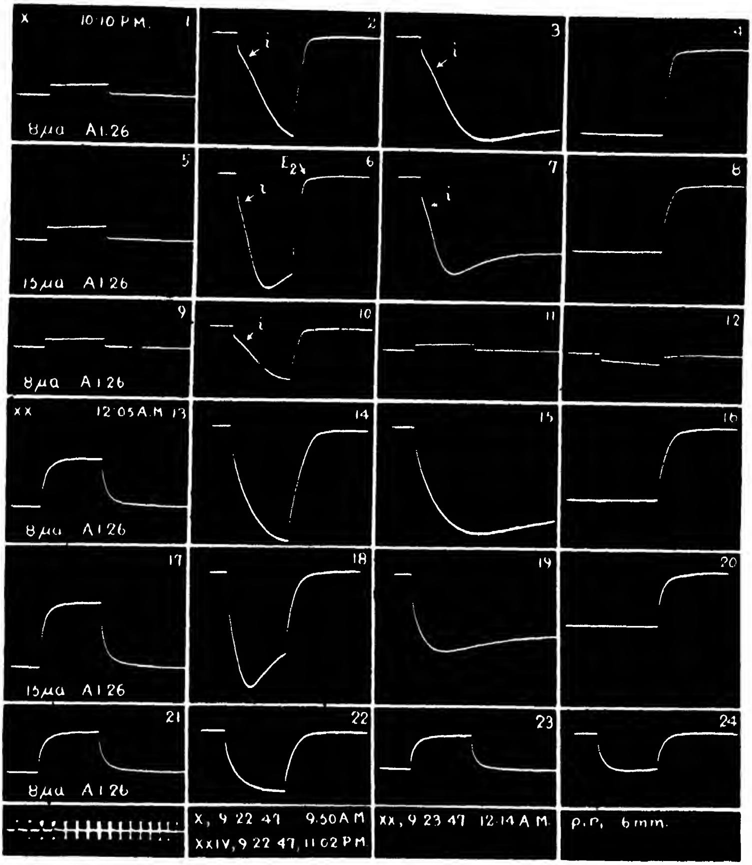


FIG. 42. Electrotonic potentials of a nerve kept in 0.11 M diethanol-dimethyl-ammonium chloride before (*1* to *12*) and after restoration by phenyl-triethyl-ammonium ions (*13* to *24*). From the same experiment as figure 20.

Records *21* and *22* were obtained, respectively, 10 and 20 seconds after the 10-second period of polarization used for records *19*, *20* and records *23*, *24*, respectively 10 and 20 seconds after a 10-second period of anodal polarization with a 40 μa current. Note that nerve reaction had caused a decrease in the value of the L fraction (cf. records *13*, *21*, *23*) and a decrease in the polarizability of the membrane by the anodal current (cf. records *14*, *22*, *24*).

2 did not decrease the polarizability of the membrane, while after the pulse of record *6* the anelectrotonus had a decreased height. Also, in those cases in which the anelectrotonus increases continuously during the flow of the applied current (cf. for example, fig. 41, *3*, *4*) no loss of polarizability of the membrane is observed. Under conditions such as these it is imperative to conclude that the loss

of polarizability of the membrane produced by the nerve reaction is an important factor in the establishment of the maximum of the anelectrotonus.

A question that prompts itself for consideration is whether or not, with nerves deprived of sodium, the lack of postanodal overshooting could be referable simply to the absence of an L fraction in the membrane potential. Indirect arguments could be offered to support an affirmative answer, but since (1) the lack of E_3 overshootings can be observed with nerves in which the L fraction has a significant value (cf. '47, fig. VII.13) and (2) the overshooting may fail to appear after the action of quaternary ammonium ions have caused the L fraction to acquire a large value (fig. 41, *10*, *12*, *14*, *16*), it seems more logical to answer the question negatively. Therefore, it must be assumed that however great the interdependence of the two results of the anodal nerve reaction may be, the loss of polarizability and the decrease in the E.M.F. of the membrane involve partly different mechanisms; a greater efficiency of the nerve reaction being necessary to produce a decrease in the E.M.F. than a decrease in polarizability of the membrane. In support of this conclusion there is also the fact that with nerve deprived of sodium no overshooting of the electrotonic potential is observed after the end of cathodal polarization, even though in this case the nerve reaction should produce an increase in the E.M.F. of the membrane and consequently an increment in the L fraction.

A phenomenon that can be observed even in advanced stages of the effect of the lack of sodium consists in a difference in the rates of establishment and of decay of the slow anelectrotonus. Even when the slow anelectrotonus grows continuously during a 10-second flow of anodal current, after the end of the applied pulse the electrotonic potential decreases practically to zero within a small fraction of a second (cf. for example, fig. 4, *3*, 4, 7, *8*; fig. 41, 3, 4). This phenomenon undoubtedly signifies that a nerve reaction has developed during the flow of the applied current; the intensity of the reaction is insufficient to cause the anelectrotonus to pass through a maximum, but it suffices to determine for the decay of the electrotonus a temporal course resembling that which is observed with normal nerve. As a matter of fact, when the potential is approaching the zero level the tracing often displays a sharp change in slope or even a definite crest (cf. arrows labelled E_2 in figs. 4, 9, 41, 42) which undoubtedly corresponds to the E_2 overshooting of the electrotonic potential of normal nerve. It was described elsewhere ('47, section XV.7) that the E_2 reaction is less dependent than the E_1 and E_2 reactions upon the availability of Na^+ ions; the observations presented here indicate that the E_2 reaction is produced with a great deal of effectiveness even in very advanced stages of the effect of the lack of Na^+ ions.

At any stage of the effect of lack of sodium the restoring quaternary ammonium ions are able to increase the effectiveness of the anodal nerve reaction. Sufficient evidence to support this statement is already a fact, that has been mentioned repeatedly: after restoring ions have acted upon the nerve anodal currents become able to create slow anelectrotonus that displays a maximum;

also important is the fact, that after the action of restoring ions the loss of polarizability after anodal polarization is very marked (cf. for example, fig. 42, *14*, *15*; *18*, *19*), but of greatest significance is the reappearance of postanodal overshootings which may result from the action of the restoring ions, since a reversal of the sign of the electrotonic potential after the end of the polarization indicates that the nerve reaction has been able to decrease the E.M.F. of the membrane. Postanodal overshootings recorded with nerves restored by quaternary ammonium ions appear in figure 4, *10*, *12*, *14*, *16*; figure 24, *10*, *12*, *14*, *16* and figure 42, *16*, *18*, *20*.

After restoration of the L fraction by quaternary ammonium ions, clear evidence is often obtained of the ability of the nerve fibers to produce the cathodal nerve reaction. In certain cases the catelectrotonus is observed to pass through a pronounced maximum during the flow of the applied current (fig. 36, *1* to *14*) and in other cases the electrotonic potential displays after the end of the applied pulse fluctuations in its value which in their general traits resemble those which are observed with normal nerve (fig. 4, *9*, *13*; fig. 36, *1*, *4*; fig. 41, *9*). However, a reversal of the sign of the electrotonic potential after the end of the polarization takes place only rarely, and when it appears the amount by which the electrotonic potential overshoots the zero level is very small. Thus, it must be stated that in nerves restored by quaternary ammonium ions the cathodal nerve reaction does not reach full development.

In view of the fact that quaternary ammonium ions are not able to restore the ability of the A fibers to conduct impulses it could not be expected that the recovery of other aspects of nerve function would be complete. It is, of course, important to know the limits of the restoring ability of quaternary ammonium ions, but special emphasis must be placed upon the positive results of the experiments, for these results serve to ascertain the mode of action of the ions upon nerve.

The theoretical significance of the effect of quaternary ammonium ions upon the electrotonic potential can be best appraised by considering the relationship that exists between the slow electrotonus and the after-potential.

On the basis of the simplifying assumption that the membrane potential has only two fractions, L and Q, the relationship of the after-potential to the electrotonic potential can be easily described (cf. '47, Chapter XIV). During conduction of a train of impulses a cumulative loss of membrane potential takes place, which is called the negative after-potential. A collapse of the L fraction during the spike of the action potential is the major factor in the production of the negative after-potential; this potential, however, also includes a deficit in the Q fraction. During the flow of an applied cathodal current a depolarization of the nerve fiber is produced, which if the magnitude of the current is appropriately chosen duplicates the negative after-potential. The changes in the value of the L fraction are the slow catelectrotonus.

After the end of the tetanus, as well as after the end of an applied pulse of cathodal current, the loss of membrane potential has to be restored by the activity of the met-

abolic mechanism of the nerve fibers, since the production of an increment in the membrane potential is a process that requires the expenditure of energy. *A priori* it could not have been predicted that the process of restoration of the loss of membrane potential produced by a cathodal current would include an increment in the resting E.M.F. of the membrane; experiment, however, has shown that after the end of an applied pulse of cathodal current the electrotonic potential reverses its sign, and detailed analysis of the phenomenon has proven that during the flow of the applied current the E.M.F. of the membane undergoes an increase (cf. '47, section VIII.4). This increase must be interpreted as the result of the operation of a regulatory mechanism, the purpose of which is to maintain the membrane potential as its resting level; the operation itself deserves the name "nerve reaction," since in increasing its E.M.F. the nerve fiber actually reacts against the effect of the applied current, i.e., against a lowering of the membrane potential.

The depolarization (negative after-potential) resulting from conduction of impulses sets into activity the same regulatory mechanism which is activated by the depolarization produced by a cathodal current. A nerve reaction develops during the tetanus, which increases the E.M.F. of the membrane; for this reason after the end of the tetanus the membrane potential increases above the resting level, i.e., the positive after-potential is produced. In the main the postcathodal overshooting of the slow electrotonus and the positive after-potential (R_3 deflection) represent increments in th value of the L fraction of the membrane potential. Detailed analysis ('47, section XV.3) has shown that the increase in the value of the L fraction is only the initial step in the recovery process; completion of the recovery requires that part of the increment in the L fraction be converted into Q fraction.

In the absence of conduction of impulses a change in the value of the membrane potential having the temporal course of the positive after-potential can be produced by applying to the nerve an anodal current. Indeed, if the magnitude of the current has been chosen appropriately, the slow anelectrotonus will duplicate the positive after-potential. This fact indicates that the flow of an applied anodal current through the membrane produces exactly the effect which results from the operation of the cathodal nerve reaction, i.e., from the activity of the metabolic mechanisms of the nerve fibers, when these mechanisms are restoring the loss of membrane potential produced by the passage of nerve impulses. The ability of an applied anodal current to restore nerve deprived of oxidative metabolism as well as nerve depressed by a variety of agents, including the lack of Na^+ ions, is obviously referable to the ability of the anodal current to initiate recovery processes, that are normally initiated by metabolic activity.

The increment in the membrane potential produced by the applied anodal current also elicits a nerve reaction that tends to oppose the effect of the current and actually causes the anelectrotonus to pass through a maximum. In the case of the anelectrotonus the nerve reaction is best interpreted as the operation of a regulatory mechanism that tends to maintain the membrane potential at the resting level. The effect of the current, i.e., the increment in the value of the L fraction is opposed by the decrease in the E.M.F. and in the polarizability of the membrane, which are produced by the nerve reaction. In the case of the positive after-potential a nerve reaction is also established which causes a decrease in the value of this potential, i.e., the transi-

tion from the R_3 to the N_3 crest. The nerve reaction elicited by the positive after-potential is exactly the same reaction that is elicited by the slow anelectrotonus; its purpose, however, is best interpreted in a different manner. The nerve reaction elicited by the positive after-potential initiates those processes by means of which the L fraction is converted into Q fraction, whereby the recovery is completed.

Since any change in the value of the membrane potential elicits a nerve reaction that opposes the change, it should be expected that the recovery of the membrane potential would have a damped oscillatory course. Actually, oscillatory behavior of the recovery process has often been recorded (cf. '47, section XV.3), but consideration of this fact is not important in the present discussion.

Quaternary ammonium ions of the restoring type are able to produce a marked improvement of the state of nerve deprived of sodium. They increase the L fraction of the membrane potential, and they restore to the nerve fibers the ability to produce nerve reactions of considerable effectiveness. In view of the significance of (1) the L fraction, (2) the electrotonic potential and (3) the nerve reaction, the action of restoring quaternary ammonium ions upon the nerve fibers can have only one interpretation. Certain metabolic processes that cannot take place or take place place in a defective manner in nerve deprived of sodium, are established again after quaternary ammonium ions are made available to the nerve; consequently, these ions must be credited with the ability to participate in chemical reactions that belong to the physiology of the nerve fiber.

d. Sodium ions. The restoration of nerve deprived of sodium by Na^+ ions is far more complete than that by quaternary ammonium ions; the manner, however, in which Na^+ ions effect the restoration indicates that the mechanism of restoration by Na^+ ions is in important respects identical with the mechanism of restoration by quaternary ammonium ions.

Figure 43 illustrates stages of the restoration of excitability by Ringer's solution of a nerve that had been kept in a 0.11 M solution of ion II for approximately 18½ hours. Records *3*, *12* and *14* prove that, as is routinely observed in experiments of this kind, the fibers of slow conduction recovered their excitability earlier than the fibers of fast conduction. The present discussion, however, is concerned only with the behavior of A fibers.

Record *1* was obtained 32 minutes after the nerve had been placed in contact with Ringer's solution; it proves that no A fibers could be stimulated to produce impulses by the closure of the 40 μa current; since no impulses were initiated even when the current was increased to 100 μa, it may be stated that the nerve fibers were inexcitable to the closure of the cathodal current. Nevertheless, a large number of fibers produced impulses in response to the break of the 40 μa anodal current (record *2*).

Essentially the same observations were made again 13 minutes later. A few A fibers were able to respond to the closure of the cathodal current (record *4*),

but many fibers were able to respond to the break of the anodal current (record *11*). This fact indicates that the majority of the responding fibers were able to produce impulses only after their excitability had been restored by the anodal current.

Records *6* to *11* are a direct proof of this conclusion. The records were obtained with the use of pulses of the 40 μa current of progressively increasing duration. As can readily be noted, the number of responding fibers increased

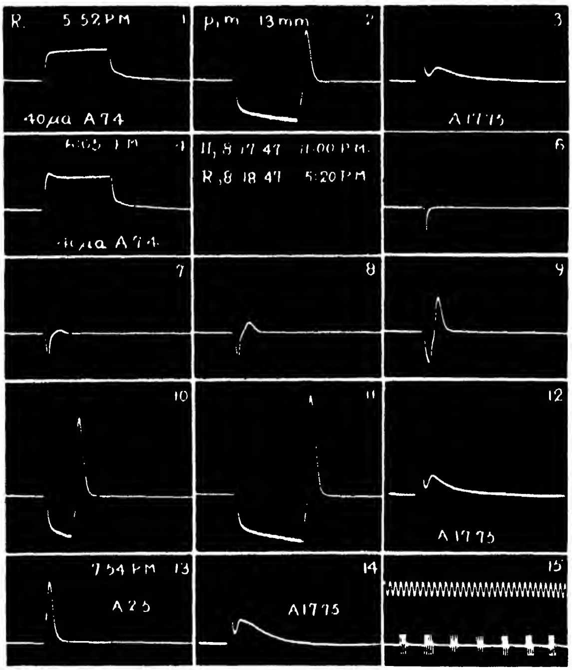


FIG. 43. Restoration by sodium of the central segment of a nerve that had been rendered inexcitable in a 0.11 M solution of ethyl-trimethyl-ammonium chloride. The lower time line (60 + 5 cy) in record *15* applies to records *3*, *12* and *14*; the upper line (1000 cy) to all the other records. From the same experiment as figures 9 and 10.

progressively with increasing duration of the applied pulse. In the case of records *6* to *9* the increase of the response may be explained in terms of the mechanism underlying break excitation; the number of responding fibers increased because lengthening of the pulse resulted in an increase of the stimulus for break excitation, the polarization counter current (cf. '47, section XII.2). In the case of records *9* to *12* the situation was different. The counter current approached its maximal value with the pulse of record *9;* consequently the large increase in the response, that is shown by records *10* and *11*, must be explained in another manner. The

explanation can be only this. The anodal current restored the excitability of a number of fibers, which increased with the duration of the applied pulse. The restoration must have begun to be significant with the pulse of record *8*, since the break response in this record is greater than the make response in record *4*.

After Na^+ ions had acted upon the nerve for $2\frac{1}{2}$ hours a large majority of the A fibers were found to be able to produce impulses in response to brief cathodal pulses (record *13*), indicating that the recovery of excitability was approaching completion.

Thus, it is clear that Na^+ ions do not restore the excitability of nerve fibers just by virtue of their presence. If the nerve has been deprived of Na^+ ions only for a relatively short period of time, the restoration of excitability takes place with such a dramatic rapidity (cf. '47, fig. I.15) that one could be inclined to believe that the excitability is restored as soon as Na^+ ions penetrate into the nerve fibers. An assumption like this is unpermissible in view of the results presented in figure 43 (cf. also '47, section I.15). The facts (1) that after prolonged absence of sodium the restoration of excitability by Na^+ ions is a slow process, and (2) that during the recovery period the applied anodal current markedly increases the number of responding fibers, these two facts lead to a different conclusion. Sodium restores excitability because in its presence chemical changes that had taken place during its absence are reversed, and it is only by long lasting activity of the metabolic mechanisms that the properties of the membrane are restored.

The recovery of excitability in the presence of Na^+ ions presents certain peculiarities if the nerve deprived of sodium has been submitted to the action of tetraethyl-ammonium ions or of any of the restoring ions with three ethyl groups.

In the experiment illustrated by figure 44 the nerve was rendered inexcitable in a solution of ion VII, and after restoration by tetraethyl-ammonium (fig. 21) the nerve was placed in contact with Ringer's solution. Records *9* to *16* of figure 44 were obtained at a stage of the recovery of excitability of A fibers, which closely corresponds to the stage illustrated by records *4* to *11* of figure 43. The closure of the cathodal current initiated impulses in a small number of fibers (fig. 44, *9*), while a large number of nerve fibers responded to the break of a 3-second pulse of anodal current (records *13*, *14*). There was, however, a significant difference between the properties of the two nerves.

In the case of the nerve used to obtain records *4* to *11* of figure 43 lengthening of the anodal pulse brought about a continuous increase in the break response. In the case of the nerve used to obtain records *10* to *16* of figure 44 lengthening of the pulse to about 5 msec also resulted in a continuous increase in the break response, but after the spike had reached the size shown in record *11*, lengthening of the pulse caused a continuous decrease in the break response, to the extent that no nerve fiber responded when the pulse was 20–30 msec long or longer

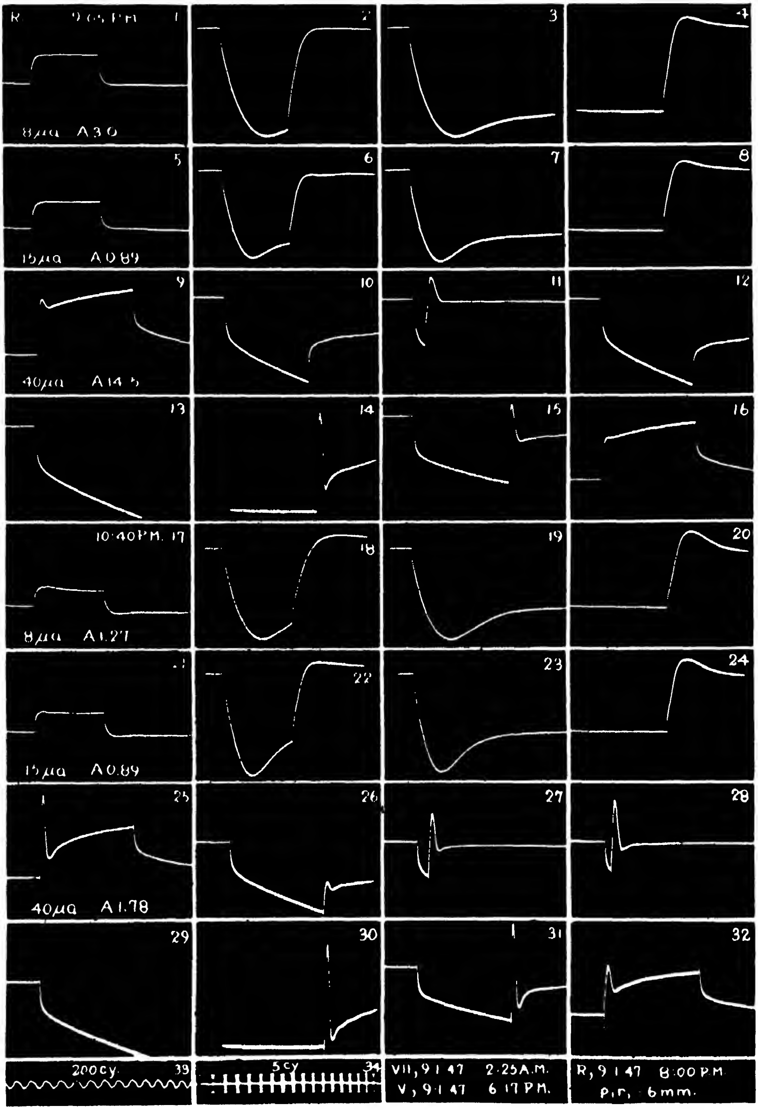


FIG. 44. Continuation of figure 21. Electrotonic potentials at two stages of the restoration by Ringer's solution. The time line in record *33* applies to records *9* to *16* and *25* to *32*; the time line in record *34*, to records *1* to *8* and *17* to *24*.

(records *10*, *12*). Nevertheless, a further lengthening of the pulse to about 300–400 msec resulted in the reappearance of the break response (records *13*, *14*); thereafter, this response increased in height with increasing duration of the

applied current, to become maximal with pulses of about one second duration. No significant difference was observed between the break responses initiated by pulses from one to 10 seconds duration. Direct observation proved that the break response began to reappear at the time when the slow anelectrotonus was reaching its maximum; the increase in the break response continued until the anelectrotonus reached its plateau; thereafter, the response remained practically constant in height. Thus, the late changes in height of the break response followed the temporal course of the slow anelectrotonus. After the end of a period of anodal polarization, the postanodal overshooting caused a temporary decrease in the value of the membrane potential (cf. base lines of records *13* and *15*), and during this period anodal pulses of 30 msec duration were able to produce large break responses (record *15*); as already mentioned, the anodal break response was observed to increase continuously in height with increasing duration of the applied pulse, in the same manner as in the case of records *4* to *11* of figure 43; the cathodal make response, however, displayed a reduced height (fig. 44, *16*).

If consideration is given to the effect that the slow anelectrotonus has upon the anodal break response (cf. '47, section XII.2) no difficulty is encountered in explaining the results illustrated by records *10* to *15* of figure 44. During the early part of its flow the anodal current restored the excitability of a number of nerve fibers, which made the break response (record *11*) greater than the make response (record *9*). The continuous increase of the slow anelectrotonus raised the threshold of stimulation of the restored fibers (cf. '47, section VI.9) to such an extent that the stimulus for break excitation, the polarization counter current did not reach the threshold of any fiber; hence, the lack of break response in record *12*. After the operation of the nerve reaction had caused the anelectrotonus to decrease below its maximal value the polarization counter current again became able to set up impulses in the restored fibers. After the end of the anodal polarization the overshooting of the electrotonic potential, i.e., the decrease in the value of the membrane potential, brought the nerve into a state of depression (cf. '47, section VIII.8), which resulted in a decrease of the cathodal make response (record *16*); the anodal current, however, was able to restore the excitability of the nerve fibers and therefore to initiate break responses. As already mentioned, the break response was observed to increase continuously in height with increasing duration of the applied current, exactly in the manner shown by records *4* to *11* of figure 43.

Thus, the difference between nerve restored by sodium directly and nerve restored by sodium after the action of tetraethyl-ammonium ions consists chiefly in that in nerve directly restored by sodium the excitability begins to reappear when the L fraction of the membrane potential has a low value, while in the case of a nerve that has been submitted to the effect of tetraethyl-ammonium ions the restoration of excitability by sodium occurs in the presence of a

large L fraction in the membrane potential. If the L fraction of the latter nerve is temporarily removed by a postanodal overshooting, the properties of the two nerves become essentially equal.

This result is theoretically important. In order to perform its recovery a nerve deprived of sodium increases the L fraction of its membrane potential (cf. '47, fig. VIII.13); if the restoration is effected directly by Na^+ ions, the increase in the L fraction is the result of the action of the Na^+ ions themselves; but if the nerve has already been restored by tetraethyl-ammonium ions, and therefore the L fraction has a large value, the action of Na^+ ions causes only an improvement of the nerve reaction. Thus, for example, a comparison of records *13* to *20* of figure 21 with records *1* to *8* of figure 44 shows that Na^+ ions had brought about an improvement of the electrotonic potential without having increased the L fraction in a readily detectable amount (cf. fig. 21, *13*, *17* with fig. 44, *1*, *5*). Signs of the improvement were, the existence of a maximum in the anelectrotonus produced by the 8μa current (cf. fig. 21, *14*, *15* with fig. 44, *2*, *3*) and the appearance of overshootings after the end of 10-second pulses of anodal current (fig. 44, *2*, *4*). The fact, however, that no overshootings were produced after the end of cathodal pulses (fig. 44, *1*, *5*) or after the end of brief anodal pulses (records *2*, *6*) was proof that the recovery of the efficiency of the nerve reaction still was in progress. After the Na^+ ions of Ringer's solution had been allowed to act upon the nerve for 95 additional minutes the appearance of postcathodal overshootings (records *17* and *21*), the sharpness of the maxima of the anelectrotonus and the large postanodal overshootings (records *18*, *20*; *22*, *24*) proved that the nerve reaction had acquired full effectiveness. Records *25* to *32* illustrate phenomena that are analogous to those which were discussed in reference to records *9* to *16*, except in one respect: the cathodal make response (record *15* was higher than the anodal break response produced by brief anodal pulses (records *27*, *28*), which is the observation routinely made with normal nerve; nevertheless, a few fibers were still in a state of depression, that was relieved by the anodal current, as is shown by the fact that the break response in record *30* was greater than the make response in record *25*. Whether or not the recovery of the depressed fibers would have become complete was not investigated.

The similarity between the restoring actions of quaternary ammonium ions and of sodium ions also is placed in evidence by observations described in preceding sections. For example, in the experiment illustrated by figures 7 and 8 restoration was effected by the successive actions of ions IV and V and of Na^+ ions; as is shown by figure 8, Na^+ ions produced an increase in the effectiveness of the nerve reaction without producing a readily detectable increment in the L fraction. At the time when the observations were discontinued, 50 minutes after the Na^+ ions had been available to the nerve, the effectiveness of the nerve reaction still was low, since the catelectrotonus displayed no overshootings

(records *13*, *17*, *21*), and the overshootings of the anelectrotonus were quite small (*14*, *16*; *18*, *20*; *22*, *24*). Comparison of records *13* to *20* of figure 8 with records *9* to *16* of figure 24 shows that in the experiment of figure 24 tetraethyl-ammonium ions had produced about the same increase in the effectiveness of the nerve reaction that Na^+ ions had produced in the experiment of figure 8. The difference was that tetraethyl-ammonium ions would not have been able to bring about further recovery, while Na^+ ions were able to produce full restoration of the nerve. A comparison of the heights of the slow catelectrotonus in records *9*, *13*, *17* and *21* of figure 24 shows that also in this case the restoration by Na^+ ions was effected without readily detectable change in the value of the L fraction.

The general conclusion to be drawn from these observations is the following. The initial part of the recovery of nerve deprived of sodium proceeds along similar lines whether the restoration is effected by tetraethyl-ammonium ions or by Na^+ ions; the final step of the recovery, however, can take place only in the presence of Na^+ ions. The conclusion can also be stated in a more definite form. The increment in the L fraction is produced by the activity of the metabolic mechanisms of the nerve fibers, in similar manners in the presence of tetraethyl-ammonium ions and in the presence of Na^+ ions; the second part of the recovery, the transformation of the increment in the L fraction into Q fraction can be successfully accomplished only in the presence of Na^+ ions.

At the present state of knowledge it is impossible to draw a detailed view of the changes in the nerve which underlie the development of inexcitability in the absence of sodium; it is possible, however, to define the problem with a certain degree of precision.

In the first place, the extrafibrillar sodium does not play any direct rôle in nerve function since the effect of the lack of Na^+ ions does not become demonstrable until the nerve has been maintained in a sodium-free medium for several hours. The rôle of extrafibrillar sodium can consist only in maintaining the osmotic equilibrium of the nerve fibers and in maintaining the internal concentration of sodium; as will be remembered, nerve kept in a sodium-free medium ultimately loses its internal sodium (cf. '47, section I.15). On the other hand, when Na^+ ions restore the excitability of the nerve fibers, the temporal course of the recovery indicates that Na^+ ions do not act instantaneously, i.e., simply by virtue of their presence; the restoration is a long lasting process during which certain changes that have taken place in the properties of the nerve fibers are reversed.

In view of these facts, and chiefly with the purpose of clarifying the concept, the rôle of the internal sodium may be compared to that of a coenzyme. When the internal sodium is lost to a sodium-free medium the activity of certain enzymatic systems becomes impaired, with the result that a progressive change in the chemical constitution of the nerve fibers takes place.

The fact that an applied anodal current may restore the properties of nerve deprived of sodium deserves emphasis. During the early stages of the effect of the lack of Na^+ ions, the anodal current may restore even the ability of the nerve fibers to conduct impulses. When the excitability is tested by determining the ability of a train of impulses to be conducted across a segment of nerve, the experimental results seem to indicate that the anodal current does not restore *the excitability of any fiber; it only restores the ability to conduct trains of* impulses to fibers that still are able to conduct single impulses (cf. '47[7], section XV.7), but when the observations are made with the technique used to obtain records *13* to *24* of figure 13 no doubt can remain that the anodal current, after it has flowed for a sufficient long interval of time, can restore the ability to produce impulses to fibers that had already become inexcitable. In the later stages of the effect of the lack of Na^+ ions the anodal current becomes unable to restore excitability; analysis of the electrotonic potential, however, reveals that even in advanced stages the anodal current may restore certain properties of the nerve fibers.

Among others the following assumption can be made in order to visualize the effect of the anodal current upon nerve deprived of sodium. The progressive decrease in the value of the L fraction of the membrane potential indicates that in the absence of sodium certain chemical species become exhausted, which are necessary for the maintenance of the L potential difference across the l–m boundary and which cannot be synthetized in the absence of sodium. The anodal current by accumulating charged particles at the l–m boundary, i.e., by creating slow anelectrotonus, initiates chemical reactions between existing reactants, which results in the synthesis of the missing species; one may even think that the polarization potential established by the anodal current supplies the energy of activation that is necessary for the chemical reactions to proceed at a measurable rate. In late stages of the effect of the lack of sodium the anodal current loses its ability to restore the properties of the nerve fibers almost completely (fig. 9). The logical assumption to explain this phenomenon is that the prolonged absence of sodium has made negligible the amounts of reactants available for the synthesis of the missing chemical species.

After sodium is made available to the nerve fibers the chemical changes, that have occurred in its absence, are reversed. To a large extent certain quaternary ammonium ions can produce the same result. This fact can be explained in two different manners. It may be assumed (1) that quaternary ammonium ions can substitute for sodium in some of those enzymatic reactions in which sodium takes part (2) that the quaternary ammonium ions may substitute for some of the products of the chemical reactions in which sodium intervenes.

The evidence that is available is not sufficient to decide between the two alternatives, but however, future research may answer the question, the fact may be regarded as established that quaternary ammonium ions of the restoring

type can participate in chemical reactions that belong to the physiology of the nerve fiber.

10. Analysis of the Effect of Tetraethyl-ammonium

This section deals with the changes in the properties of nerve fibers kept in a 0.11 M solution of tetraethyl-ammonium chloride for long periods of time. The study has considerable theoretical interest.

It was mentioned in section 3,a that restoration by tetraethyl-ammonium ions of the ability of Et fibers to conduct impulses was accompanied by important changes in the duration of the spike, in the speed of conduction and in the rate of recovery after conduction. Similar changes are induced by all the other restoring ions. The immediate conclusion to be drawn from this fact is that the restoring ions, when they substitute for Na^+ ions, induce changes in the chemical constitution of the nerve fibers, that are important but compatible with the accomplishment of nerve function.

Tetraethyl-ammonium ions occupy a special position among all the other restoring ions, since (1) in nerves kept in a 0.11 M solution of tetraethyl-ammonium chloride the Et fibers remain excitable practically as long as in nerves kept in Ringer's solution and (2) in nerves that have been rendered inexcitable in a 0.11 M solution of one of the inert ions and then have been restored by tetraethyl-ammonium ions the Et fibers remain excitable for many hours, while the other restoring ions render the Et fibers inexcitable after they have acted upon the nerve for a certain number of hours, that varies with the different ions. In other words, among all the restoring quaternary ammonium ions known at present tetraethyl-ammonium is the best substitute for sodium. The properties of Et fibers kept in 0.11 M tetraethyl-ammonium chloride for long periods of time will be analyzed in subsection b.

Although no quaternary ammonium ion is able to restore to A fibers deprived of sodium the ability to conduct impulses, the restoring ions improve the state of the membrane. They produce an increment in the L fraction and an increase in the effectiveness of the nerve reaction. In section 8,c and d, the effect of the restoring ions and in particular that of tetraethyl-ammonium was regarded as being essentially identical with the first step in the restoration by Na^+ ions. This simplification was permissible because the restoring ions were allowed to act upon the nerve for short periods of time only. The true state of affairs is that the restoring ions when they substitute for sodium induce changes in the nerve fibers that in part must be reversed by the action of sodium. If the restoring ions act only for a short period of time the changes may be considered as a step toward the normal state of the nerve; if the ions, however, are allowed to act for a long period of time the changes progress to the extent of causing important abnormalities in the state of the nerve.

That the restoring ions exert upon the A fibers actions of their own, i.e., ac-

tions that are not referable to the lack of Na^+ ions was clearly shown by the results presented in section 8. All the restoring ions have a property in common, they increase both the L fraction and the L/Q ratio. With the majority of restoring ions these changes ultimately result in a decrease in the membrane potential; ions XII, IX and V, however, maintain the total value of the membrane potential at the normal level. Thus, the changes in the constitution of the membrane induced by these three ions are compatible with the accomplishment of a certain aspect of nerve function. The most profound changes are induced by tetraethyl-ammonium.

It is advisable to study first the properties of the A fibers of nerves kept in 0.11 M tetraethyl-ammonium chloride.

a. A fibers. The recording of the electrotonic potentials reproduced in figures 45 and 46 describe the state of the A fibers of nerves that had been kept in tetraethyl-ammonium chloride for $22\frac{1}{2}$ hours (fig. 45) and $24\frac{1}{2}$ hours (fig. 46). The two series of records, *1* to *12* of figure 45 and *5* to *16* of figure 46, are virtually identical. The records of the catelectrotonus (fig. 45, *1, 5, 9;* fig. 46, *5, 9, 13*) present large slow components, that passed through maxima during the flow of the applied current; the dependence of the maxima upon the magnitude of the applied current also was essentially that which is observed with normal nerve; an increase in the magnitude of the current resulted in an earlier appearance as well as in a sharpening of the maximum. These two facts indicate that the L fraction of the membrane potential had a high value and that a cathodal nerve reaction was established during the flow of the applied current. The nerve reaction, however, was established in a defective manner, since after the end of the polarization the electrotonic potential did not overshoot the zero potential level by a readily detectable amount. On the other hand, the height of the slow catelectrotonus was much greater than that which is observed with normal nerve. In the case of a freshly excised nerve in an atmosphere of 95 % O_2 and 5 % CO_2, i.e., a case in which the L fraction has almost the largest possible value for untreated, resting nerve, the height of the slow catelectrotonus produced by 8 or 15 μa currents would have been only a small fraction of the height of the fast electrotonus.

Records *1* to *4* of figure 46 demonstrate in an interesting manner that the L fraction had an exceedingly high value. The records were obtained with the use of pulses of current of progressively increasing duration. With the pulses used for records *1* to *3* the slow electrotonus displayed a rising phase after the end of the applied current; a rising phase can be observed even in record *4*. This phenomenon is a consequence of the peculiarities of the electrotonic propagation of changes in the membrane potential in nerves in which the L fraction is large; with the values of the L fraction which prevail in normal nerve a rising phase in the electrotonic potential after the end of the applied current can be observed only at a relatively great distance from the polarizing electrode (Davis

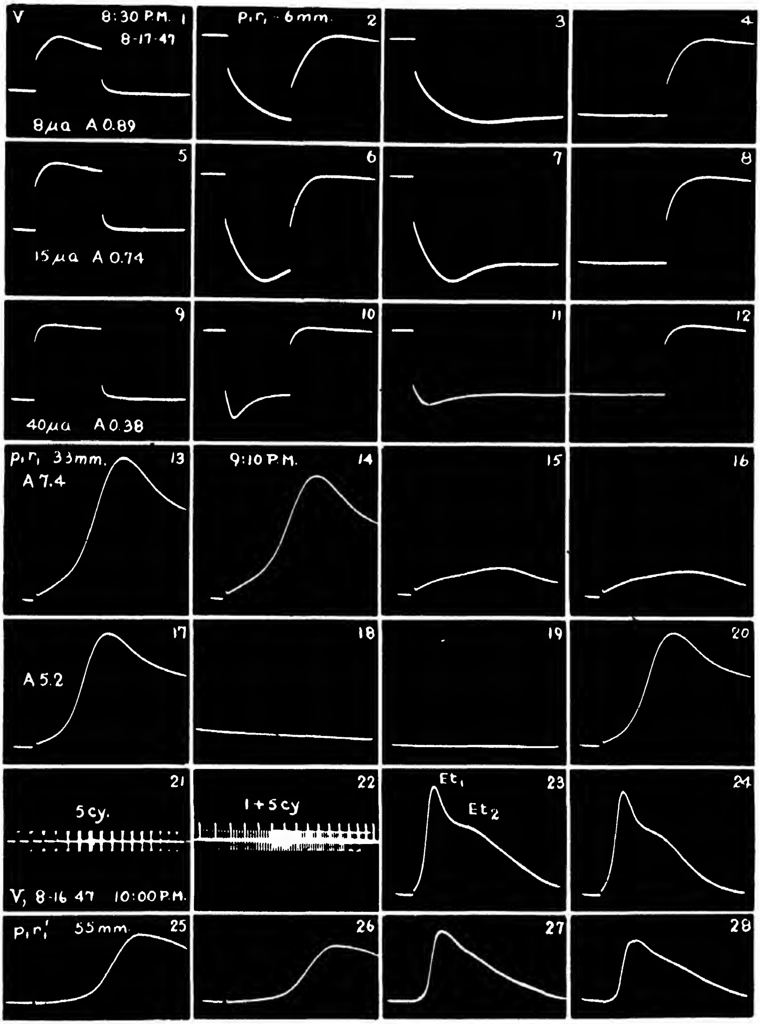


FIG. 45. Electrotonic potentials (*1* to *12*) and conducted responses (*13* to *28*) in a nerve kept in 0.11 M tetraethyl-ammonium chloride. Time line *22* applies to records *23*, *24* and *27*, *28*; time line *21* to all the other records. The interval between records *13* and *14* was 30 seconds; between the records of the series *14* to *16*, *17* to *20* and *25*, *26*, 10 seconds; between records *23*, *24* and between records *27*, *28*, 30 seconds.

and Lorente de Nó, '47, fig. 28) or from a conduction block ('47, fig. XIV.2). The appearance of such a rising phase (fig. 46, *1* to *4*) at a short distance (6 mm) from the polarizing electrode indicates that the L fraction has a very high value.

A cursory examination of the records of the anelectrotonus (fig. 45, *2* to *4*; *6* to *8*, and *10* to *12*; fig. 46, *6* to *8*; *10* to *12*, and *14* to *16*) will fail to detect

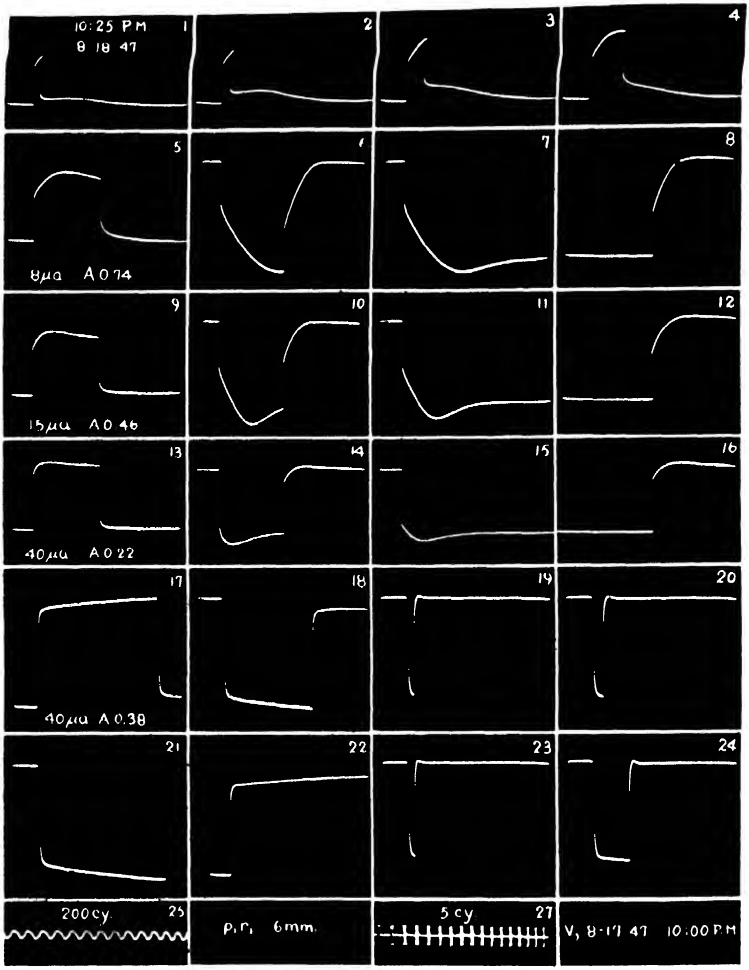


FIG. 46. Electrotonic potentials in a nerve kept in 0.11 M tetraethyl-ammonium chloride. Time line *25* applies to records *17* to *24*; time line *27* to all the other records.

gross abnormalities, since the anelectrotonus passed through maxima during the flow of the applied current and reversed its sign after the end of the polarization. Detailed analysis, however, proves the existence of important abnormalities. In the first place the rate of establishment and the height of the maximum of the anelectrotonus were much smaller than those which would have

been observed with nerves kept in Ringer's solution. Thus, despite the high value of the L fraction, the polarizability of the membrane was low; a remarkable behavior, since usually the polarizability of the membrane increases with increasing value of the membrane potential (cf. '47, Chapter VII). On the other hand, the postanodal overshootings (fig. 45, *2*, *4*; *6*, *8*; *10*, *12*; fig. 46, *6*, *8*; *10*, *12*; *14*, *16*) were very small, which was a proof of defective establishment of the anodal nerve reaction.

Thus, it appears that tetraethyl-ammonium ions and the inert quaternary ammonium ions alter the constitution of the membrane potential in essentially different manners. In nerves kept in 0.11 *M* tetraethyl-ammonium chloride the L fraction has an abnormally high value, and consequently, since the total value of the membrane potential is normal, the Q fraction has a subnormal value. A nerve reaction is elicited by cathodal as well as by anodal currents in qualitatively normal fashion, since the electrotonic potentials display maxima and overshootings; the effectiveness of the reaction, however, is impaired, as is shown by the small size and the low rate of establishment of the overshootings. The abnormal constitution of the membrane potential may be regarded as a direct consequence of the lack of effectiveness of the nerve reaction. Tetraethyl-ammonium ions replace Na^+ ions and enable the metabolic mechanisms to create L potential, but the process underlying the transformation of L fraction into Q fraction, i.e., the anodal nerve reaction, takes place in a defective manner. Remarkably enough, the constitution of the membrane potential in nerves kept in 0.11 M tetraethyl-ammonium ions closely resembles that which exists in nerves that have been depolarized by veratrine and have been allowed to repolarize themselves in the presence of 5% CO_2 (cf. '47, sections II.7, XIII.1d and XV.6).

The changes in the properties of the nerve fibers induced by long lasting action of tetraethyl-ammonium are reversible by sodium, but at an exceedingly low rate. Figure 47 illustrates observations made with a nerve that had been kept in 0.11 M tetraethyl-ammonium chloride for 16½ hours. Records *1* to *8* were obtained before placing the nerve in contact with Ringer's solution and records *9* to *16*, 4 hours after Na^+ ions had been made available to the nerve. In the presence of Na^+ ions two changes occurred in the electrotonic potential. Comparison of records *1* and *9* and *5* and *13* shows that the slow catelectrotonus had decreased in height, or otherwise stated that the L fraction of the membrane potential had decreased. On the other hand, the effectiveness of the nerve reaction had increased markedly, as is shown by three facts: (1) no overshooting appears in record *5* and only a small and late one in record *1*, while relatively large overshootings appear in records *9* and *13*; (2) the maxima of the anelectrotonus are sharper in records *10*, *11* and *14*, *15* than in records *2*, *3* and *6*, *7*; (3) the overshootings of the anelectrotonus are greater in records *10*, *12* and *14*, *16* than in records *2*, *4* and *6*, *8*.

Although at the time when records *9* to *16* were obtained Na^+ ions had been available to the nerve for 4 hours the recovery was not yet complete. In the first place the great height of the catelectrotonus in records *9* and *13* indicates that the L fraction of the membrane potential still had an abnormally high value; on the other hand, there still were a number of A fibers that could produce impulses only in response to the break of the anodal current, i.e., after their excitability had been restored by the anodal current.

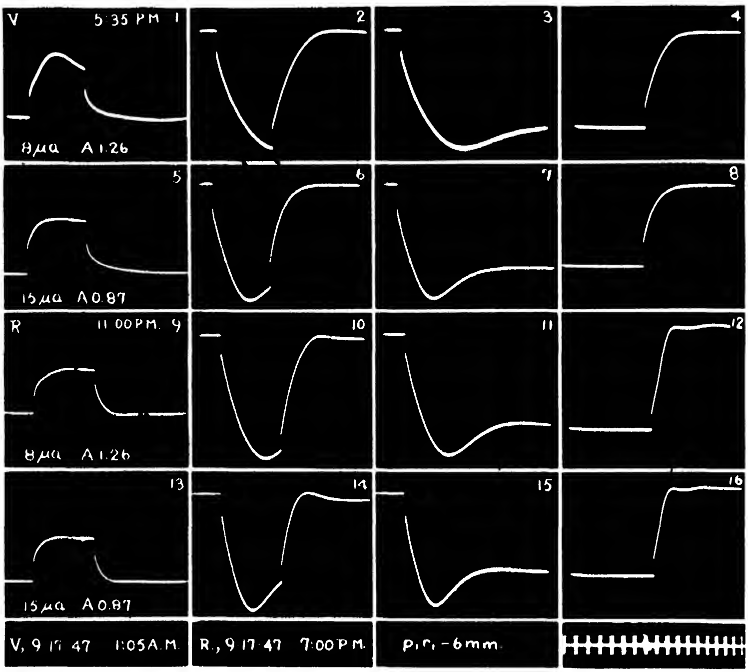


FIG. 47. Electrotonic potentials in a nerve kept in 0.11 M tetraethyl-ammonium chloride, before (*1* to *8*) and after restoration by Ringer's solution (*9* to *16*).

Emphasis may be placed upon a difference that exists between the restoration of excitability by Na^+ ions of nerves deprived of sodium in a solution of one of the inert ions and the restoration of nerves kept in a solution of tetraethyl-ammonium ions. The difference has already been described in section 9,d in reference to figures 43 and 44, for the case of a nerve that had been in the presence of restoring ions for only a few hours. With a nerve kept in tetraethyl-ammonium chloride until inexcitability develops, the A fibers recover in Ringer's solution the ability to produce impulses in response to the break of the anodal earlier than the ability to respond to the closure of the cathodal current; this is also the behavior of nerves kept in solutions of inert ions, but while in these

nerves the restoration is rapidly completed, nerves treated with tetraethyl-ammonium perform their recovery at an exceedingly low rate. For example, in the experiment illustrated by figure 6 a number of A fibers were able to respond to the break of the anodal current 20 minutes after Na^+ ions had been made available to the nerve, and 20 minutes later a large majority of the A

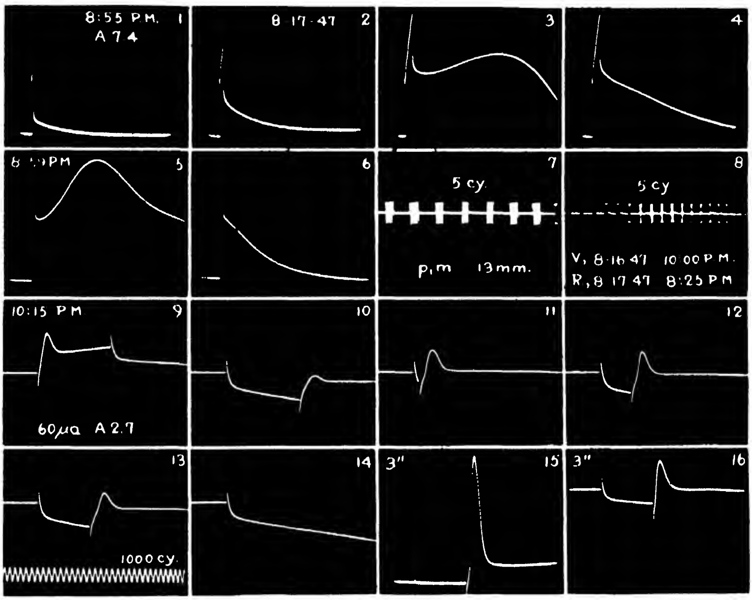


FIG. 48. Initial stages of the restoration by Ringer's solution of the central segment of a nerve kept in 0.11 M tetraethyl-ammonium chloride. Time line *7* applies to records *1* to *4*; time line *8*, to records *5* and *6* and the time line below record *13*, to records *9* to *16*. The intervals between records *3*, *4* and *5*, *6* were 10 seconds and the intervals between the records of the series *9* to *16*, three seconds.

fibers were able to respond to a brief cathodal pulse. In contrast with this behavior, in the experiment illustrated by figure 50, after 36 minutes of the action of Na^+ ions, very few fibers responded to the closure of the cathodal current (record *1*) and only a discrete number responded to the break of the anodal current (records *2*, *4*). A slightly more advanced stage of the recovery is illustrated by records *9* to *16* of figure 48. After Na^+ ions had acted upon the nerve for 110 minutes a number of A fibers were able to respond to the closure of the 60 μa cathodal current (record *9*) but the number of fibers that responded to the break of a 3-second pulse of anodal current (records *14*, *15*) was much greater. The spike of record *15* did not include all the A fibers. In all probability, one additional hour would have been necessary for all the A fibers to become excitable to the break of the anodal current.

The slow rate of the recovery of excitability corresponds to the slow rate of the return to normality of the electrotonic potential, or otherwise stated of the transformation of L into Q fraction. Judging by their ability to produce impulses in response to applied currents the A fibers display during the recovery the behavior which should be expected from partly depolarized fibers. An observation like this would be paradoxical, since the total value of the membrane potential is normal, were it not that an important abnormality exists in the constitution of the membrane potential. As already mentioned the L fraction of the membrane potential is higher than in normal nerve, while the Q fraction has a subnormal value; thus, since the behavior of the mechanism underlying the production of the nerve impulse is directly determined by the value of the Q fraction (cf. '47, section III.5, XIV.6, XV.3 and XV.8) nerve treated with tetraethyl-ammonium must behave like partly depolarized nerve until the L/Q ratio approaches the normal value.

Two details that appear in records *17* to *24* of figure 46 deserve comment. The sweep speed used to obtain these records was relatively high; consequently, in records *17*, *18*, *19* and *20* the height of the deflections at the make and at the break of the applied currents, up to the time when the tracings display a sharp change in slope, measure the height of the fast electrotonus, i.e., the height of the discontinuities in the records obtained at low sweep speed (cf. '47, section VI.3). In the case of the short pulses used to obtain records *17* and *18* the fast deflections were approximately equal at the beginning and at the end of the applied pulse; in the case of the 3-second pulse used to obtain records *21* and *22* the fast deflection at the end of the polarization was smaller than that at the start of the applied pulse. On the other hand, records *23* and *24* show that the fast deflection at the start of the polarization also had a decreased height, indicating that a long lasting change in the properties of the membrane had been produced. This behavior of the fast anelectrotonus is precisely the opposite of that which is illustrated by records *7* and *8* of figure 9. If consideration is given to the effect that a reduction in the value of the L fraction has upon the rate of establishment of the electrotonus (cf. '47, fig. XII.9) it becomes clear that a difference in the heights of the two fast deflections in records *21* and *22* of figure 46 must have been the result of the operation of regulatory mechanisms of the membrane during the flow of the applied current.

In this connection it is important to mention that with nerves that are being kept in solutions of the inert ions, before the stage illustrated by records *1* to *8* of figure 9 is reached, the fast deflections at the start and at the end of long anodal pulses are approximately equal, while after restoration of the nerve by tetraethyl-ammonium ions the two fast deflections are unequal in the manner shown by records *21* and *22* of figure 46. Although not so pronounced the phenomenon also appears with nerves kept in choline chloride (fig. 14, *9* to *12* and *21* to *24*). Detailed analysis of the phenomenon will undoubtedly contribute to the understanding of the difficult problem of the nature of the fast electrotonus (cf. '47, section VIII.3).

The second detail consists in that although no A fibers were able to conduct impulses a few A fibers produced impulses in response to the opening of brief pulses of anodal

current (fig. 46, *19*, *20*; *23*, *24*). This phenomenon does not appear only with nerves kept in tetraethyl-ammonium chloride; examples of it observed with nerves kept in 0.11 M choline chloride have been presented in figures 13 and 14. Records *25* to *27* of figure 9 illustrate the phenomenon in the case of a nerve that had been kept in a 0.11 M solution of ethyl-trimethyl-ammonium chloride. It will be noted that treatment of the nerve first with dimethyl-diethyl ions (record *26*) and then with tetraethyl-ammonium ions (record *27*) did not increase the number of responding fibers; as a matter of fact the presence of the restoring ions did not prevent a decrease in the

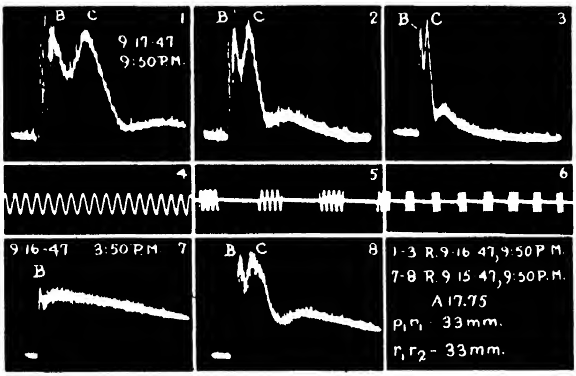


FIG. 49. Spikes of the fibers of slow conduction ((B, C) in nerve kept in Ringer's solution. Note that a few fibers of the A group were firing impulses in the absence of stimulation.

spike height. In a few additional hours the break response would have ceased to appear. Thus, it must be concluded that among the A fibers there is a small group that is particularly resistant to the effect of the lack of Na^+ ions; ultimately, however, also these fibers become inexcitable, and as is the case with all other A fibers, quaternary ammonium ions are not able to restore their excitability.

b. Fibers of slow conduction. Figure 49 illustrates the action potential of the fibers of slow conduction of nerves that had been kept in Ringer's solution for 18 hours (records *7*, *8*) and 24 hours (records *1* to *3*). The spike height and the shock spike times can serve as standards to determine the effects of tetraethyl-ammonium chloride in other experiments. Since the A fibers were on the verge of the rhythmic state—as a matter of fact, a few fibers were firing impulses spontaneously—the rectangular pulses of current used to stimulate the fibers of slow conduction initiated a violent repetitive firing by A fibers, which resulted in a slight distortion of the B elevation. On the other hand, it should be noted that the B and C elevations were superposed upon the decay of the negative after-potential of the A fibers. Finally, it should be mentioned that the second recording electrode was placed on a point of responding nerve, 3 mm

before the killed end; however, since the interelectrode distance was 30 mm, the height of the first phase of the spike was not decreased by the arrival of the impulses to the second electrode. The spike heights that appear in figure 49 are of the same order of magnitude as the spike heights that are recorded with freshly excised nerves.

Figure 45, *13* to *28* presents Et spikes of a nerve that had been kept in tetraethyl-ammonium chloride for 23 hours. The conduction distance was 33 mm for records *13* to *24*, and 55 mm for records *25* to *28*, but since the central half of the nerve had already been in contact with Ringer's solution for 45 minutes the conduction time in the initial 13 mm was practically negligible in relation to the conduction time in the rest of the p_1r_1 and $p_1r'_1$ segments. Although the sweep speed used for records *13* to *20* and *25*, *26* was quite low (cf. record *21*) the Et spike proved to be longer than the sweep deflection; in order to include the whole spike within the limits of the screen of the oscillograph (records *23*, *24*; *27*, *28*) it was necessary to reduce the sweep speed greatly (record *22*). If the difference between the amplifications used is taken into account, a comparison of records *23*, *24* of figure 45 with the records of figure 49 shows immediately that tetraethyl-ammonium ions had produced a very large increase in the height of the spike of the fibers of slow conduction; accurate figures cannot be calculated because the temporal dispersion of the individual fiber spikes was different in the two cases; nevertheless, since the height of the Et_1 elevation in record *23* of figure 45 is about 4 times the height of the B or C elevation in figure 48, it may be concluded that under the influence of tetraethyl-ammohium ions the individual fiber spikes had become several times, probably three or 4 times higher than the spikes in a freshly excised nerve or in a nerve kept in Ringer's solution.

The second effect of tetraethyl-ammonium ions consists in a tremendous increase in the spike duration. The amount by which the duration of the individual fiber spikes was increased cannot be ascertained by a direct comparison of records *23*, *24* of figure 45 with the records of figure 49; the direct comparison, however, leaves no doubt that the increase had been very great. A conclusive proof of the ability of tetraethyl-ammonium ions to increase the duration of the spike of fibers of slow conduction will be presented below (fig. 52).

The third effect of tetraethyl-ammonium ions consists in a tremendous reduction in the speed of conduction. An accurate measurement of the speed of conduction on the basis of the records of figure 45 is not possible, since in the case of records *13* and *17* the spike arose from the rising phase of the electrotonic potential produced in the A fibers by the stimulating current; the direct comparison, however, of records *13* to *24* of figure 45 with the records of figure 49 leaves no doubt that in the nerve treated with tetraethyl-ammonium ions the speed of conduction was only a small fraction of the speed of conduction in normal nerve. A more accurate comparison will be made below, in reference to figure 50.

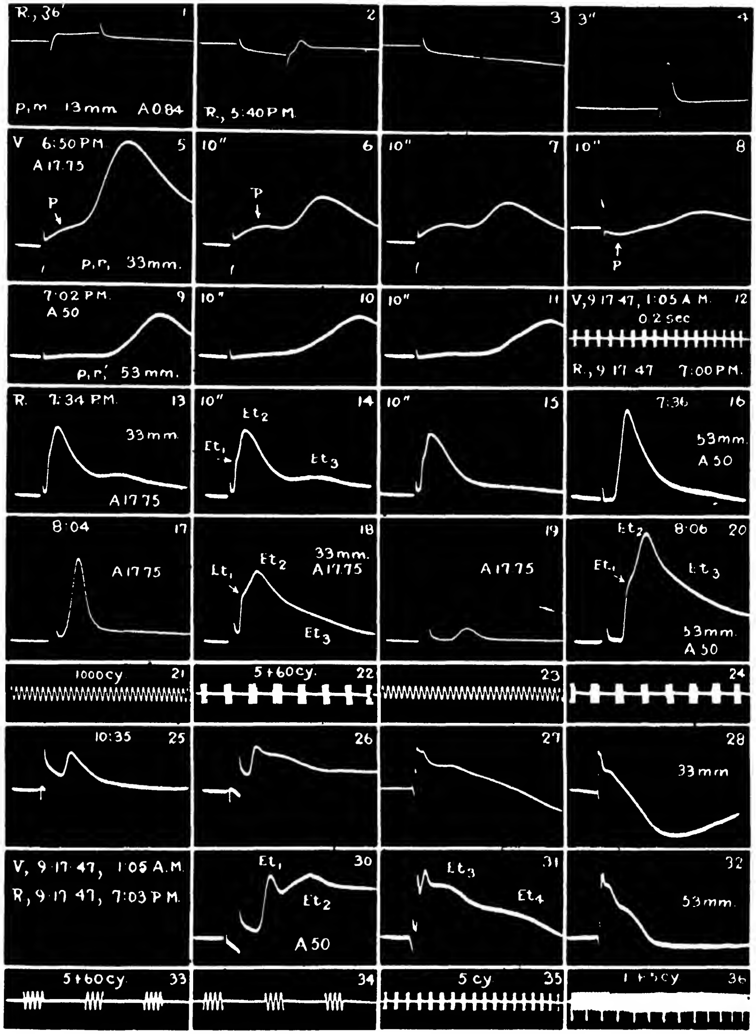


FIG. 50. Restoration by Ringer's solution of a nerve kept in 0.11 M tetraethylammonium chloride.

1 to *4*, initial stages of the restoration of the A fibers in the central segment. Ringer's solution was applied to this segment at 5:40 P.M. and record *1* was obtained at 6:16 P.M. with the use of a 40 μa current.

5 to *11*, conducted responses in the peripheral segment of the nerve, recorded at two different points. *P*, electrotonic potential in the A fibers.

13 to *32*, stages of the restoration by Ringer's solution.

Time line *21*, *23*, applies to records *1* to *4* and *17*, *19*; time line *12* to records *5* to *16*; time line *22*, *24*, to records *19*, *20* and time lines *33* to *36* to the corresponding records of the series *25* to *32*. Records *25* to *28* were obtained at the same amplification (A17.75) as records *5* to *8*, *13* to *15*, and *17* to *19*.

The 4th effect of tetraethyl-ammonium ions consists in a tremendous increase in the fatigability of the nerve fibers, or otherwise stated in a tremendous reduction of the rate of recovery after conduction. With the nerves used to obtain the records of figure 49 the fibers of slow conduction (B, C) were able to conduct volleys of impulses at 3-second intervals, without readily detectable signs of fatigue, for long periods of time. In the case of the nerve treated with tetraethyl-ammonium ions a slight but readily detectable decrease in the spike height was produced when the stimulation was applied at 30-second intervals (fig. 45, *13*, *14*; *23*, *24*; *25*, *26*; *27*, *28*). When, as was the case with records *14* to *16*, the stimulation was applied at 10-second intervals, only a few fibers were able to follow the stimulus, indicating that the absolutely refractory period of many of the Et fibers was longer than 10 seconds.

Records *17* to *20* illustrate this situation in an instructive manner. The records were obtained at 10-second intervals, but stimulation was applied only in the case of records *17* and *20*; the zero potential level is common to the 4 records. It will be noted that 10 seconds after the spike of record *17*, the negative after-potential still had a relatively large value (record *18*); the residual negativity decreased during the following 10 seconds (record *19*) and disappeared almost entirely in 10 additional seconds. After the disappearance of the residual negativity had become nearly complete the spike displayed a height (record *20*), that was only slightly smaller than with the resting nerve (record *17*), while when the stimulation was applied during the negative after-potential the spike had a markedly reduced height (record *15*). Thus, the residual depolarization (negative after-potential) left by the passage of the nerve impulse was the immediate cause of the inexcitability of many, and of the high threshold of stimulation of the other fibers. As was explained elsewhere ('47, section XV.8), the negative after-potential always is a sign of depression, even when the excitability of the nerve is exalted ("supernormal"), since the negative after-potential is a sign, as well as a measure, of incomplete recovery of the membrane potential. In the particular case that is now under consideration the depression was so great that many of the nerve fibers were unable to conduct impulses.

The effects of tetraethyl-ammonium upon the Et fibers is reversible by the action of Na^+ ions. It will be useful to describe first a phenomenon of considerable theoretical as well as practical importance. If the progress of the recovery is tested in the segment of nerve in which the impulses are being initiated the recovery seems to proceed at a lower rate than when the recovery is followed in terms of the ability of nerve impulses to propagate themselves.

For example, records *1* to *6* of figure 48 were obtained from the central segment of the nerve, after this segment had been in contact with Ringer's solution for 30 minutes. The durations of the rectangular pulses of current used for records *1* and *2* were not sufficient to stimulate any Et fiber, so that these

records present only the electrotonic potential produced in A fibers by the applied currents; a rising phase in the slow catelectrotonus did not appear after the end of the applied pulses because the presence of Na^+ ions had already resulted in a significant decrease in the L fraction of the membrane potential and therefore the rising phase could be observed only at greater distances from the polarizing electrode. The duration of the pulse used to obtain record *3* was sufficient to initiate impulses in a number of Et fibers, the spikes of which were superposed upon the decay of the electrotonic potential of the A fibers; 10 seconds later the same pulse was able to initiate hardly any Et impulse (record *4*). Finally, record *5* was obtained with a pulse of duration sufficient to stimulate all the Et fibers; 10 seconds later hardly any Et fiber was able to respond to the same pulse (record *6*). These results were obtained again after the applied current had been increased from 100 to 200 μa.

In view of these observations it would seem logical to conclude that the absolutely refractory period of the majority of Et fibers was longer than 10 seconds. The conclusion, however, would be erroneous. If the ability of the Et fibers to conduct impulses is tested after the recovery of the central segment of the nerve is well advanced, it is found that a number of Et fibers are able to conduct impulses in the peripheral segment at 10-second intervals (fig. 50, *5* to *11*) and that in 30 minutes Na^+ ions are able to reduce markedly the fatigability of the Et fibers (fig. 50, *13* to *15*). Thus, the correct conclusion to be drawn from the observations presented in figure 48, *1* to *6* are that with nerves in tetraethylammonium, as well as during the initial stages of the recovery in Ringer's solution, nerve impulses cannot be initiated during the early part of the relatively refractory period by rectangular pulses of current, even though they may be initiated by the action current. Since an increase in the magnitude of the applied rectangular current does not alter the experimental results, the difference between the effects of the flow of the applied current and of the flow of the action current must be referable solely to differences in the spatial distribution and in the temporal course of the two currents. Similar problems have been discussed elsewhere ('47, section XII.1c); for the purpose of the present discussion it is sufficient to emphasize the existence of conditions under which a nerve may be inexcitable to applied cathodal currents but capable of conducting impulses. It has been in consideration of this fact that in the research presented here one full hour has been allowed for the recovery of the central segment in Ringer's solution before testing the ability of the peripheral segment to conduct impulses.

A systematic analysis is presented in figure 50, *5* to *32* of the recovery of the peripheral segment in the presence of Na^+ ions. The observations were begun 70 minutes after the central segment had been placed in contact with Ringer's solution. Records *5* to *8* illustrate the state of Et fibers kept in tetraethylammonium for 17 hours. In record *5* the Et spike arises from the maximum of

the electrotonic potential (*P*) produced in the A fibers by the stimulating current; in the following records *6* and *7* the electrotonic potential and the Et spike appear as distinct deflections because the speed of conduction of the Et fibers had been markedly reduced by repetitive activity; in record *8* the electrotonic potential appears with reversed sign because electrode p_1 was made the anode of the stimulating current. In records *9* to *11* that were obtained with the use of a greater conduction distance the electrotonic potential is almost undetectable. From a comparison of records *5* and *9* it follows that the fastest fibers were conducting impulses at the speed of 50 mm per second.

Records *13* to *16* illustrate the important changes in the Et spike that were observed 34 minutes after Na^+ ions had been made available to the nerve. Comparison of records *5* to *7* with records *13* to *15* and of record *9* with record *16* shows that (1) the spike height had decreased markedly and (2) the conduction time had become a small fraction of its initial value; on the other hand, records *13* to *15* show that the fatigability of the nerve fibers had decreased. The Et spike displayed three distinct elevations labelled Et_1, Et_2 and Et_3 in record *14*; the three elevations became indistinct during further propagation of the impulses (record *16*).

A more advanced stage of the recovery is illustrated by records *17* to *20*. Fibers of the A group had begun to conduct impulses (records *17* and *19*) although at the low speed of 10 m per second, and the speed of conduction of the Et fibers had increased so far that the observations had to be continued with the use of a relatively high sweep speed in the oscillograph (records *22*, *24*). The three elevations in the Et spike were detectable at the two points at which records were obtained (records *18*, *20*). In record *20* the spike displays a greater height than in record *16*; the increase, however, was referable to a diminution of the effect of temporal dispersion of the individual fiber spikes, since in record *18* the spike is not higher than in record *13*. At the time when records *18* and *20* were obtained the speed of conduction of the fibers of the Et_1 elevation was approximately 200 mm per second.

The recovery progressed with advancing time although at a steadily decreasing rate. In order to analyze in detail the properties of the Et spike after 215 minutes of the action of Na^+ ions records *25* to *32* were obtained at three different sweep speeds (records *33* to *36*). The Et spike displayed 4 distinct elevations labelled Et_1 to Et_4 in records *30* and *31*. That the Et_1 elevation corresponds to the B elevation of normal nerve is shown by record *25* that was obtained with the use of a pulse of duration chosen so that only B fibers could be stimulated to conduct impulses. Comparison of records *26* and *30* shows that the Et_1 elevation was conducted at the speed of 600 mm per second. Further evidence of the return toward normality effected by Na^+ ions was the decrease in the spike height; in records *26* to *32* the spike height was nearly that which is observed with nerve kept in Ringer's solution (fig. 49). Particular attention

deserves the large negative after-potential that appears in records *27* and *31* and the large positive after-potential that appears in records *28* and *32*. The great magnitude of the after-potential indicates that the L fraction of the membrane potential still was abnormally great, which is in agreement with the observations made with A fibers (fig. 47, *9*, *13*). Although not the only one (cf. below), the large value of the L fraction undoubtedly was a cause of the reduced speed of conduction (cf. '47, section III.4).

Thus, the results of the experiment illustrated by figure 50 leaves no doubt that in the presence of Na^+ ions the changes induced in the Et fibers by tetraethyl-ammonium ions are reversed and that the reversal is the result of processes that take place at a low rate. Experiments of the type of that which is illustrated by figures 51 and 52 have yielded complementary information.

The experiment of figures 51 and 52 was done with the two sciatic nerves of a bullfrog. After they had become inexcitable in a 0.11 M solution of ion X, the nerves were mounted in identical manners in two different moist chambers. Ringer's solution was applied at 3:10 P.M. to the whole nerve I (fig. 51), and to the central segment of nerve II (fig. 52) a few seconds later.

The recovery of nerve I is illustrated by figure 51. Essentially the same results would have been observed if the nerve had been rendered inexcitable in a solution of saccharose; thus, the recovery consisted only in the reversal of changes produced by the absence of Na^+ ions. The observations were made with the use of two conduction distances, 33 and 53 mm. The second recording electrode (r_2) was placed on the end of the nerve and for the reason that this end had been created many hours before the spikes were recorded with large diphasic artifacts. As has been customary in the research presented here, the nerve fibers were stimulated to conduct impulses by rectangular pulses of cathodal current (100 μa), the strength of the stimulation being varied by changing the duration of the applied pulse. The stimulus is maximal for the A group when the pulse is a fraction of 1 msec long; it becomes maximal for the fibers of the C group when the duration of the pulse is of the order of 30-40 msec. The strength of the stimulus is given in arbitrary units (st.1, st.2, etc.) with the records of figures 51 and 52; the units are the divisions of the dial of one of the two potentiometers that serve to regulate the duration of the pulse. The zero setting in the second potentiometer corresponds to a strength of stimulation which is slightly greater than maximal for the A group of fibers. The use of rectangular pulses of current to stimulate fibers of slow conduction prevents damage to the fibers of fast conduction; on the other hand, it introduces no significant complications in the observations, since the current used is far above the rheobase of all but the fibers of highest threshold (cf. '47, section XII.1).

A small number of A fibers became able to conduct impulses 20 minutes after Na^+ ions had been made available to the nerve (record *1*). The speed of conduction was exceedingly low, 5 m per second, but the speed of conduction as

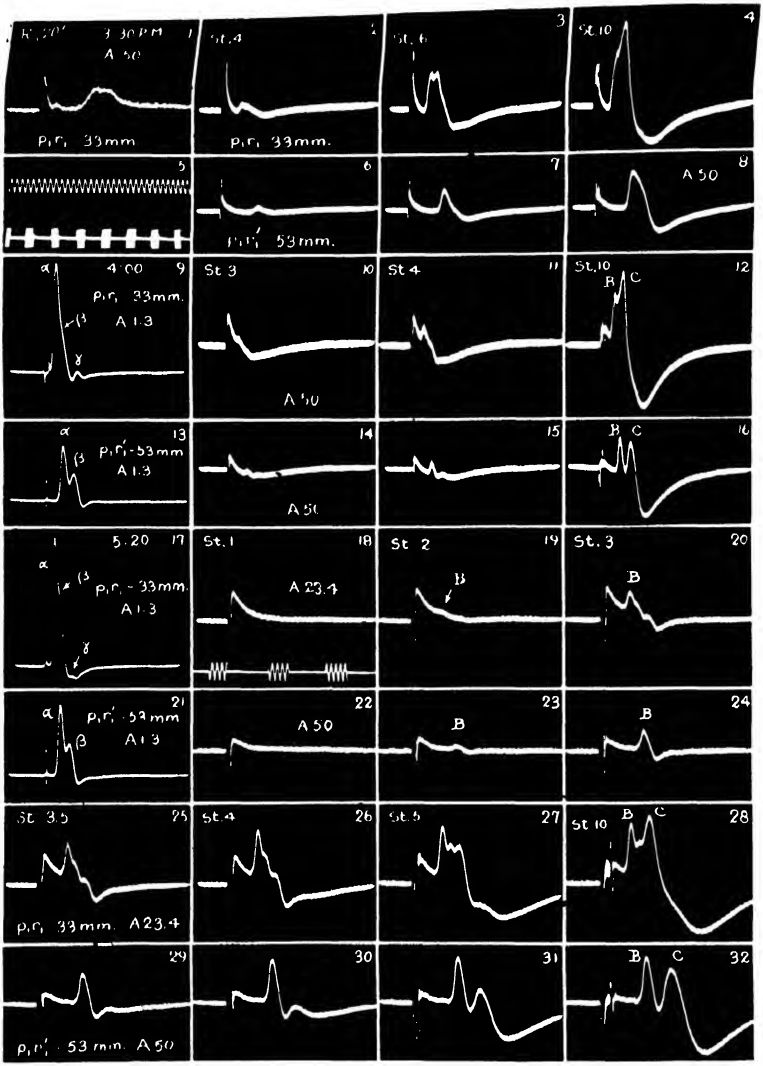


FIG. 51. Restoration by Ringer's solution of the excitability of a nerve that had been rendered inexcitable in a 0.11 M solution of diethanol-dimethyl-ammonium. The nerve was placed in this solution at 10:30 P.M., the day before the observations were made and was treated with Ringer's solution 20 minutes before record *1* was obtained.

The upper time line (1000 cy) in record *5* applies to records *1*, *9*, *13*, *17* and *21*; the lower time line (5 cy) in record *5*, to records *2* to *4*, *6* to *8*, *10* to *12*, and *14* to *16* and the time line below record *18* to records *18* to *20*, *22* to *24*, and *25* to *32*. The stimulus strengths (st.4; st.6, etc.) apply to the two records of each pair (p_1r_1 33 mm and p_1r_1 53 mm).

well as the number of conducting fibers rapidly increased during the following 30 minutes. At the time when records *9* and *13* were obtained a large majority of A fibers were able to conduct and the speed of propagation of the fastest impulses was approximately 25 m per second. The recovery was complete at the time when records *17* and *21* were obtained; the speed of conduction of the fastest fibers was approximately 36 m per second which is practically the normal speed of conduction. The A spike presented three elevations, α, β, γ, that undoubtedly correspond to the three elevations that bear the same denomination in Erlanger and Gasser's classification of A fibers (cf. Erlanger, '37).

The recovery of the fibers of slow conduction progressed more rapidly than that of the fibers of fast conduction. The heights of the spikes in records *4* and *8* indicate that after 20 minutes of the action of Na^+ ions a large majority of the fibers of slow conduction were able to conduct impulses. On the other hand records *2* and *6* prove that among the restored fibers there were fibers of the B group; it is true that after a longer action of Na^+ ions the stimulus used to obtain records *2* and *6* became able to stimulate some C fibers, but during the initial stages of the recovery the threshold of stimulation was so high that the stimulus could not have initiated impulses in C fibers. In records *4* and *8* the spike does not appear divided into B and C elevations and the speed of conduction of the fastest fibers was quite low, 100 mm per second.

A later stage in the recovery is illustrated by records *10* to *12* and *14* to *16* that were obtained at the same sweep speed as records *2* to *4* and *6* to *8* (record *5*, below). The progress in the recovery was noticeable chiefly in the decrease in the threshold of stimulation and in the increase in the speed of conduction. The increase was observed with all fibers, but it was relatively greater with the B than with the C fibers, which resulted in a fractionation of the spike in two distinct elevations (records *12*, *16*; B, C). The separation of the two elevations became more pronounced with advancing time, until the stage illustrated by records *18* to *20*, *22* to *24* and *25* to *32* was reached; probably this stage corresponded to the optimum of the recovery.

A sharp discontinuity existed between the fibers of fast and those of slow conduction, since no detectable increase in the number of responding fibers was observed when the strength of the stimulus was varied between that which was used for records *17* and *21* and that which was used for records *19* and *23*. Since the latter strength was not greater than that which usually suffices to stimulate low threshold B fibers in normal nerve it may be concluded that the spike labelled B in records *19* and *23* corresponds to the B elevation of the compound spike of normal nerve.

Particular attention deserves the fact that the speed of conduction of the restored B fibers was quite low, about 0.65 m per second, as against about 3 m per second in normal nerve (cf. fig. 49). Thus, since the speed of conduction of the A fibers (records *17* and *21*) was practically normal, it is obvious that the

results of the recovery from the effect of the lack of Na^+ ions established a significant difference between the A and the B fibers, the counterpart of which is that at least a large number of B fibers belong to the Et class.

Although the evidence is perhaps not entirely conclusive it is probable that a discontinuity exists between the A and B groups in normal nerve. Erlanger ('37) regards the fibers of the A and B groups as belonging to a continuous series because the compound spike presents a rather inconstant elevation (δ) bridging the gap between the A and B elevations. The present writer, however, has never been able to rule out the possibility that the elevation is referable to repetitive discharge of fibers of the α and β groups. On the other hand, Gasser ('41) believes that in the case of mammalian nerves the A and B groups are discontinuous.

According to the fairly complete series of records presented in figure 51 no discontinuity exists between the B and C elevations, since progressive strengthening of the stimulus caused a progressive increase in the number of responding fibers until the C elevation reached its maximal height. In the experience of the present writer a similar situation prevails in the case of normal nerve. The lack of discontinuity between the B and C elevations makes it impossible to give a definite figure as the maximal speed of conduction of C fibers. The main C elevations of records *28* and *32* yields 0.25 m per second as a representative figure for the speed of the fastest fibers contributing to the elevation. This figure is low in relation to the similar figure, about 0.85 m per second, which is determined with normal nerve (fig. 49); it should be noted, however, that after recovery from the effect of the lack of Na^+ ions the speeds of conduction of the fibers contributing to the B and C elevations were reduced by approximately the same factor.

For the purpose of the present discussions it is important to mention that after completion of the recovery the speeds of conduction of the B and C elevations of records *28* and *32* of figure 51 were approximately equal to the speeds of conduction of the Et_1 and Et_2 elevations of records *26* and *30* of figure 50. Thus, the low value of the speed of conduction of Et fibers in the experiment illustrated by figure 50 (*28*, *32*) was not wholly referable to a specific effect of tetraethyl-ammonium ions; to a large extent it was a peculiarity of the recovery of Et fibers deprived of sodium, which is not dependent upon the sodium-free medium in which the Et fibers have become inexcitable. On the other hand, the low value of the speed of conduction of A fibers restored by sodium, which is observed when the nerve has been submitted to the effect of tetraethyl-ammonium ions (cf. fig. 18, *29*, *30*; fig. 30, *33*, *35*), probably is entirely the result of the increase in the value of the L fraction of the membrane potential which is produced by tetraethyl-ammonium and removed only at an exceedingly low rate by the action of sodium.

Another detail worthy of emphasis is that the spike height was approximately

equal in the case of records *26* to *28* of figure 50 and in that of record *28* of figure 51. In both instances the spike height was approximately equal to that which is observed with normal nerve (fig. 49).

In the case of nerve II (fig. 52) the recovery of the central segment in the presence of Na^+ ions progressed in the same manner as the recovery of nerve I. Records *1* to *8* of figure 52 were obtained 60 minutes after the nerve had been placed in contact with Ringer's solution. The recovery of the A fibers (record *1*) as well as that of the fibers of slow conduction (records *2* to *8*) was in an advanced stage. Since the conduction distance was only 13 mm the spikes were not fractionated into distinct elevations. It should be noted that the maximal B + C spike was recorded at two different sweep speeds (records *7*, *11*; *8*, *12*), which were later used for records *25* to *32* and records *33* to *40*.

The peripheral segment of the nerve (fig. 3, II, mr_2) was placed in contact with 0.11 M tetraethyl-ammonium chloride at 4:30 P.M., which resulted in a rapid recovery of the ability to conduct impulses by fibers of the Et class. Records *13* to *16* of figure 52 that were obtained with the use of stimuli of progressively increasing strength can serve to establish a correspondence between the B and C groups of normal nerve and the Et class. A conducted response can be observed in record *13* that was obtained with the use of a stimulus capable of initiating impulses only in fibers of the B group and a conducted response appears also in record *14* in spite of the fact that the stimulus used could not have reached the threshold of C fibers. Thus, there can be no doubt that fibers of the B group belong to the Et class. In the case of record *16* there is no doubt that the Et spike included a large number of fibers of the C group; nevertheless, the spike in record *16* differs from that in record *12* only in magnitude; the temporal courses of both spikes are equal. Thus, there is a remarkable fact, the B fibers restored by tetraethyl-ammonium ions conduct impulses in the manner of the restored C fibers, in spite of the differences that with normal nerve exist between the two groups of fibers. Similar results are illustrated by records *17* to *23*. Corresponding to the lowering of the threshold in the central segment of the nerve the threshold Et spike was elicited by a slightly weaker stimulus in the case of record *18* than in the case of record *17;* on the other hand the progress of the recovery of the peripheral segment had resulted in an increase of the maximal Et spike (cf. records *16* and *23*); the essential fact, however, remained unchanged: strengthening of the stimulus resulted only in slight changes in the temporal course of the Et spike, indicating that although this spike included responses of B and C fibers, all the restored fibers were conducting impulses in approximately the same manner. The counterpart of this important fact is the progressive fractionation of the Et spike which is observed during the recovery in Ringer's solution of nerve kept in tetraethyl-ammonium (fig. 50). The manner in which the fractionation occurs clearly shows that the speed of conduction of the B fibers increases relatively much more than that of the

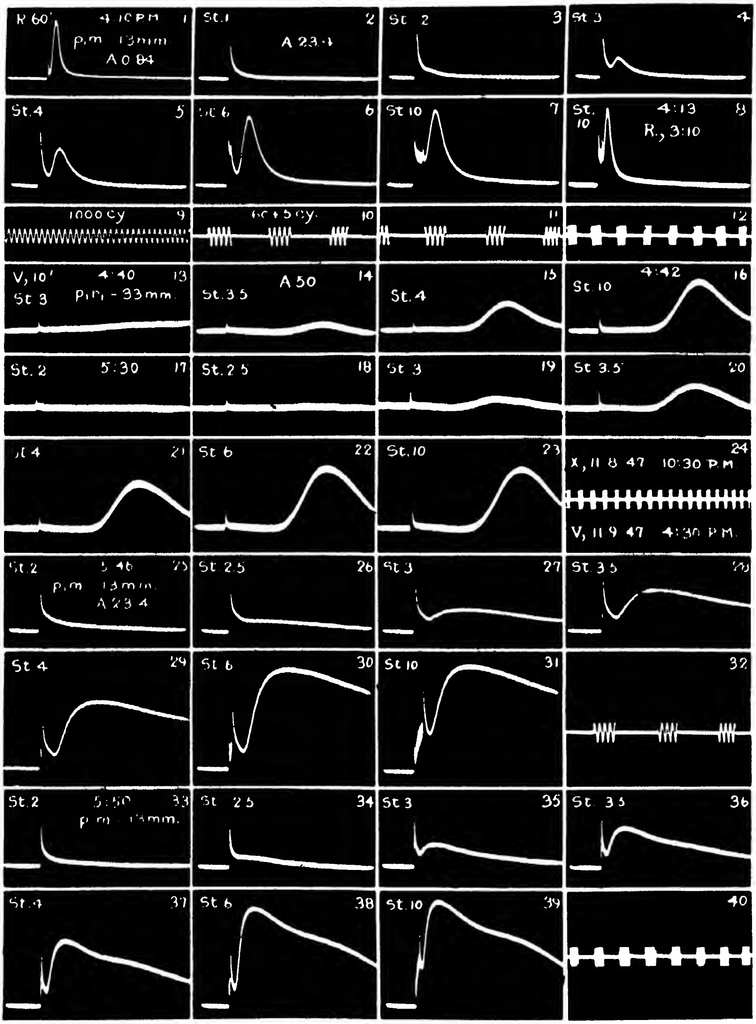


FIG. 52. Restoration of the excitability of a nerve rendered inexcitable in 0.11 M diethanol-dimethyl-ammonium chloride. From the same experiment as figure 51.

1 to *8*. Conducted responses recorded at point m after restoration of the central segment by Ringer's solution. Time line *9* applies to record *1*; time line *12*, to record *8*, and time lines *10*, *11*, to records *2* to *7*.

13 to *23*, conducted responses in the peripheral segment at two stages of the restoration by tetraethyl-ammonium ions. Time line *24* applies to records *13* to *23*.

25 to *31* and *33* to *39*, spikes of the fibers of slow conduction recorded at point m after the action of tetraethyl-ammonium ions. Time line *32* applies to records *25* to *31*; time line *40*, to records *33* to *39*.

C fibers. A similar phenomenon appears in figure 51, indicating that also during the recovery from the effect of lack of Na^+ ions of nerves kept in "inert" solutions, the B and C fibers have comparable speeds of conduction during the initial phases of the restoration.

In order to demonstrate in a convincing manner the effect that tetraethyl-ammonium ions have upon the magnitude and the duration of the spike of fibers of slow conduction the observations illustrated by records *2* to *8* were repeated after the tetraethyl-ammonium ions had acted upon the nerve for 76 minutes, i.e., records *25* to *31* and *33* to *39* were obtained with the first recording electrode (r_1) at the margin (m) between the central and the peripheral segment of the nerve and the second recording electrode (r_2) at the end of the nerve (fig. 3,II). Since the central segment of the nerve had been kept in contact with Ringer's solution, the conduction of impulses up to the immediate proximity of point m must have taken place in the same manner as in the case of records *2* to *8;* therefore, temporal dispersion cannot have been a significant factor in determining the shape of the spikes that appear in records *25* to *31* and *33* to *39;* in other words these spikes may be regarded as a true measure of the changes induced by tetraethyl-ammonium in the individual fiber spikes at point m. A comparison of the records obtained with the use of stimuli of comparable strength leaves no doubt that tetraethyl-ammonium ions had caused an important increase in the spike height and a tremendous increase in the spike duration, the two effects being present both in the B and in the C fibers. Thus, there can be no doubt that tetraethyl-ammonium ions had caused important changes in those electrochemical reactions which underlie the production of the alteration or nerve impulse.

c. Comment. It will contribute to the clarity of the discussion to mention here that in order to produce its characteristic effect upon the nerve fibers, tetraethyl-ammonium need not be present at the 0.11 M concentration; for example, nerves kept in a 0.132 solution of saccharose with tetraethyl-ammonium chloride at the concentration of 0.044 M undergo essentially the same changes as nerves kept in 0.11 M tetraethyl-ammonium. On the other hand, the presence of Na^+ ions does not prevent the action of tetraethyl-ammonium. A drastic proof of this statement is given by the experiment illustrated by figure 52. The central segment of the nerve including point m, and of course the neighboring 1 or 2 mm of the peripheral segment had performed their recovery in Ringer's solution; during the brief period of time (76 minutes) that point m and the next 1 or 2 mm of the central segment were in contact with the tetraethyl-ammonium solution, the effect of the lack of Na^+ ions in the external medium would have been hardly, if at all detectable if an inert sodium-free medium had been used; nevertheless, tetraethyl-ammonium produced the spectacular changes in the Et spike, that are recorded in figure 52, *25* to *39*.

A similar observation can be made with freshly excised nerve. A few hours

after the nerve has been placed in a sodium-free solution of tetraethyl-ammonium chloride (0.11 M), i.e., long before the effect of the lack of Na^+ ions would have been detectable with the use of an inert sodium-free medium, the *A fibers that still are excitable, become unable to conduct impulses as a result of a large increase in the L fraction of the membrane potential*, and the *B* and C fibers conduct impulses at the reduced speed which is so characteristic for nerves treated with tetraethyl-ammonium. An observation like this indicates either that tetraethyl-ammonium ions have replaced the internal Na^+ ions or that the internal Na^+ ions cannot prevent the action of tetraethyl-ammonium. The second alternative would be in agreement with the fact, mentioned in section 9,b that tetrabutyl-ammonium ions have been observed to produce their action upon nerve at concentrations as low as 0.011 and 0.022 M, i.e., in the presence of Na^+ ions at the concentrations of 0.099 and 0.088 M. The second alternative also is in agreement with another experimental fact. The presence of Na^+ ions at the 0.022 M concentration does not prevent the action of tetraethyl-ammonium, in spite of the fact that addition of Na^+ ions at the 0.022 M concentration to a solution of saccharose is sufficient to prevent the development of inexcitability of the nerve.

Finally, the fact must be mentioned that the low speed of conduction and the great duration of the spike in nerves treated with tetraethyl-ammonium are characteristic traits of the action of the substance. It is true that a decrease in the speed of conduction is observed before the nerve fibers become inexcitable in any inert sodium-free medium (cf. fig. 15), but conduction ceases at a time when the speed of conduction is much greater than in nerve treated with tetraethyl-ammonium (cf. fig. 50); likewise, a lengthening of the spike duration is produced by several agents (cf. '47, section XV.5a), but only in the case of a lowering of the temperature almost down to the inexcitability limit, is the lengthening of the spike comparable to that which is produced by tetraethyl-ammonium.

Under conditions such as these it is unavoidable to conclude that the active, quaternary ammonium ions, and in particular tetraethyl-ammonium ions exert their action upon nerve because they participate in those chemical reactions, which underlie the establishment of the resting membrane potential and that of the nerve impulse. Whether the active quaternary ammonium ions exert their action by replacing the Na^+ ions or by replacing products of chemical reactions in which Na^+ ions take part, this is a question that cannot be answered with assurance solely on the basis of the available evidence. The essential fact, however, is that certain quaternary ammonium ions, indeed no less than 15 ions, have chemical properties resembling those of substances naturally occurring in the nerve fibers to the extent that they may participate in the accomplishment of nerve function. To state that this observation may open new lines of research in nerve physiology is perhaps not to voice undue optimism.

A certain amount of emphasis may be placed upon the difference that exists between the A and the Et fibers. No direct information is available on the intimate nature of the difference, but it seems logical to believe that the difference is of chemical nature. On the other hand, the evidence indicates that each class includes several smaller subdivisions. In the case of the A fibers there are considerable variations in the resistance to the effects of the lack of Na^+ ions (cf. end of section 2, section 4,b and end of section 10,a). In the case of the Et fibers there are important differences in the intensity of the effects of tetraethyl-ammonium (cf. section 10,b) as well as of other restoring ions (cf. sections 4,b and 5,b and c). The existence of subclasses in the Et class often is revealed by the fractionation of the Et spike into several elevations. A number of examples of fractionated Et spikes have been presented in the illustrations, even though they have not always been mentioned explicitly in the text.

Important as it would be to decide whether or not all the fibers of the B and C group in Erlanger and Gasser's classification belong to the Et class, the evidence does not justify a definitive statement. The results of experiments of the type of those which are illustrated by figures 50 to 52 certainly suggest that if not all, at least the majority of the B and C fibers belong to the Et class; nevertheless the possibility cannot be excluded that a number of B and C fibers become inexcitable in tetraethyl-ammonium chloride.

Finally, attention will be called upon a noteworthy fact. Existing knowledge makes the conclusion virtually certain that the fibers that contribute to the B elevation in the compound spike of untreated nerve or at least a large majority of them are myelinated, their diameters ranging from 2 to 6–7 μ (cf. Erlanger, '37, fig. 11). The Et class, therefore, includes both myelinated and unmyelinated fibers.

11. Effect of Thiamine

As was mentioned in section 1 thiamine chloride cannot be used to prepare a sodium-free medium because at the 0.11 M concentration thiamine causes a depolarization of the nerve fibers. This fact, however, does not exclude the possibility that thiamine could be a restoring ion, since many restoring ions are depolarizing agents. Therefore, it seemed advisable to investigate whether or not thiamine can restore the excitability of nerve fibers deprived of sodium.

a. Experimental facts. The effect of thiamine upon nerve deprived of sodium has been analyzed in one experiment only, because the results were so decisive that it seemed unnecessary to repeat the observations.

The experiment was done with the use of the two sciatic nerves of a bullfrog, that were rendered inexcitable in a 0.11 M solution of diethanol-dimethyl-ammonium chloride. After the central segment of the nerves had performed successful recoveries in Ringer's solution the peripheral segments were placed in contact with 0.11 M solutions of thiamine chloride. In the case of nerve I the

solution was applied after the peripheral segment had been in the sodium-free medium 18 hours; in the case of nerve II, 19 hours.

Since the 0.11 M solution of thiamine chloride (Merck) is strongly acid (pH below 3) barium hydroxide was added to raise the pH. According to available information on the effect of barium upon nerve (Lorente de Nó and Feng, '46), during the relatively short time that the test solutions were kept in contact with the nerves, the presence of Ba^{++} ions could not be expected to introduce any complication in the experiment. The pH of the solution used for nerve I was adjusted to 6 and that of the solution for nerve II, to 6.7. According to available information changes in the pH of the external medium within the range of 5.5–8 by themselves have little effect upon nerve ('47, section III.7).

In the case of nerve I the thiamine solution was allowed to act for 40 minutes and in that of nerve II, for 60 minutes. In both cases thiamine proved to be unable to restore the excitability of any nerve fiber; nevertheless, 0.11 M tetra ethyl-ammonium chloride produced in both cases a rapid and complete recovery by the Et fibers of the ability to conduct impulses, exactly as if the nerves had not been treated with thiamine chloride. Ringer's solution produced, also in both cases, a complete recovery of the A fibers.

b. Comment. The immediate conclusions to be drawn from the experimental results are: (1) when thiamine is allowed to act upon the nerve for no longer than one hour it fails to produce damage to the nerve fibers and (2) thiamine is not able to substitute for sodium, or otherwise stated, thiamine does not belong to the group of restoring, quaternary ammonium ions.

It may be of interest to note that although the group of known restoring ions is quite large, it does not include any of the three ions at present known to be a constituent of nervous tissue, choline, acetylcholine and thiamine.

12. Statement of a Hypothesis

The observations presented in this paper constitute additional evidence to support the view that the resting membrane potential is not directly dependent upon the existence in the external medium of the nerve fibers of any of the ionic species normally present in blood serum ('47, section I.14 and Concluding Notes). Nerve fibers kept in artificial media, such as a 0.22 M solution of saccharose or a 0.11 M solution of one of the inert quaternary ammonium ions, are self-contained mechanisms, or stated in a figurative language, biological machines that create and maintain their membrane potential by transformation of metabolic substrates that they contain, with the use of atmospheric oxygen. On the other hand, none of the solutes normally present in blood serum, not even oxygen (cf. '47, section XV.4), is immediately necessary for the production of the nerve impulse. The maintenance of the excitability, however, requires that two elements be available to the nerve, oxygen and sodium. The rôle played by oxygen is indirect: anoxic nerve becomes inexcitable be-

cause it undergoes depolarization (cf. '47, Chapter XIII); whether or not sodium plays a direct rôle is a more difficult question to answer.

The loss of excitability by nerve deprived of sodium is a slow process that as a rule requires 14–16 hours for completion. This fact by itself does not answer the question, since the loss of the intrafibrillar sodium probably is a slow process. More revealing is the fact that also the recovery from the effect of prolonged lack of sodium is a slow process. Since in relation to the duration of the restoration process the penetration of Na^+ ions into the nerve may be regarded as instantaneous, the logical assumption is that sodium restores the excitability because in its presence certain chemical changes are reversed, which had taken place in its absence. Probably, therefore, the rôle played by sodium in maintaining the excitability of the nerve fibers is only indirect; to clarify the concept, the rôle of sodium may be compared to that of a coenzyme (cf. section 9,d).

To a large extent certain quaternary ammonium ions can substitute for sodium. When acting upon nerve deprived of sodium the restoring ions restore the excitability of the fibers of the Et class, i.e., numerically the more important class of nerve fibers, and they restore important properties to the other fibers (the A group). Moreover, many quaternary ammonium ions have proved to exert important effects upon the mechanism that maintains the resting membrane potential; several ions are powerful depolarizing agents and the action of some of the depolarizing ions has been observed to proceed to the extent that the nerve fibers lose their core conductor properties, which indicates that the membrane of the nerve fibers has undergone disintegration. The detailed analysis presented in sections 9 and 10 has led to the conclusion that a number of quaternary ammonium ions are able to participate in chemical processes that belong to the physiology of the nerve fiber.

Under conditions such as these it seems permissible to state a working hypothesis capable of filling the vacuum left by the disqualification of Bernstein's hypothesis on the rôle that potassium would play in nerve physiology. This hypothesis, that in the past had played so important a rôle, had to be abandoned, because it proved to be inadequate (cf. '47, section I.14); a formal concept was elaborated to explain the nature of the resting membrane potential (cf. '47, Concluding Notes), but the available evidence did not justify assumptions regarding the nature of the electric double layers in the membrane. The assumption to be made now is that quaternary ammonium ions directly participate in the maintenance of the resting membrane potential and in the production of the nerve impulse.

The facts, (1) that the number of active quaternary ammonium ions is large—indeed, no less than 15 restoring ions are known at present—and (2) that substitution of one of the groups attached to nitrogen may be sufficient to radically alter the properties of an ion, these two facts indicate that the

attention should be directed to the properties of nitrogen rather than to the properties of specific quaternary ammonium ions. In other words, the observations presented in this paper should not be interpreted in relation to the rôle that could be played by specific compounds such as, for example, choline, acetylcholine or thiamine; the results should be interpreted in a more general manner.

Compounds containing trivalent nitrogen are weak bases while the introduction of a 4th group, linked to nitrogen by covalency, results in an enormous increase in basicity and consequently in the appearance of electrovalent linkages. For this reason a chemical reaction that results in the change of an amine into a quaternary ammonium base is a reaction that results in the creation of ions, i.e., in the appearance of charged particles of opposite signs, where previously electrically neutral compounds were present. Thus, since trivalent nitrogen is an abundant constituent of nerve tissue and since quaternary ammonium ions have proved to be able to participate in the physiology of the nerve fiber, the following assumption may be made.

Chemical reactions which result in a change of tricovalent nitrogen into tetracovalent nitrogen participate in the establishment of electric double layers in the nerve membrane.

13. SUMMARY

A study has been made of the effect on frog nerve of 24 simple quaternary ammonium ions, that are listed in figures 1 and 2. The groups attached to nitrogen were: (1) combinations of methyl, ethyl and ethanol groups, (2) three ethyl groups and one group of the series n-propyl to n-hexyl or the phenyl, the β-phenylethyl or the acetyl-β-hydroxyethyl group and (3) 4 n-propyl or 4 n-butyl groups. Often it was found that substitution of one group was sufficient to produce a radical change in the properties of the ions.

Certain ions, tetramethyl-ammonium, ethyl-trimethyl-ammonium and diethanol-dimethyl-ammonium are inert, since in 0.11 M solutions of these ions the nerve fibers maintain their membrane potential at the normal level and become inexcitable in the same manner as in other, inert sodium-free media (0.22 M saccharose). Except for slight differences nerves kept in solutions of choline chloride behave in the same manner as nerves kept in the solution of an inert ion.

A number of quaternary ammonium ions exert a depolarizing action upon the nerve fibers. Figures 38 to 40 present demarcation potential curves obtained with the use of 23 different ions. In some cases the depolarizing action goes so far that the nerve fibers lose their core conductor properties. The mechanism of the depolarization is discussed in section 9.

Nerves kept in a 0.11 M solution (sodium-free) of tetraethyl-ammonium chloride maintain their membrane potential at the same level as nerves kept

in Ringer's solution; the fibers of the A group lose their excitability as they would in other sodium-free media but fibers of slow conduction remain excitable practically as long as in nerves kept in Ringer's solution. The fibers that remain excitable in 0.11 M tetraethyl-ammonium chloride have been grouped in a special class, Et; if not all, at least the majority of the fibers of groups B and C, i.e., myelinated as well as unmyelinated fibers, belong to the Et class. Certain modifications of the action potential of Et fibers and of the electrotonic potential of A fibers indicate that tetraethyl-ammonium ions are able to participate in chemical reactions that belong to the normal physiology of the nerve fiber.

The excitability of Et fibers that have become inexcitable in sodium-free media can be restored by quaternary ammonium ions that contain two or more ethyl groups. The restoring ability of an ion is not in direct relation to its effect on the resting membrane potential, although all the restoring ions have the property in common to increase, temporarily at least, the L fraction of the membrane potential. The fact is noteworthy that nerve fibers that have become inexcitable in ethanol-trimethyl-ammonium (choline) recover their excitability when ethanol-triethyl-ammonium ions are made available to them. Acetylation of its alcohol group reduces the restoring ability of ethanol-triethyl-ammonium.

The effectiveness of the restoring ions, as measured by the number of fibers that recover the excitability and by the rapidity of action, increases with the number of ethanol and more so with the number of ethyl groups present in the ion. Tetraethyl-ammonium is the best substitute for sodium, but phenyl-triethyl-ammonium, n-propyl-triethyl-ammonium, n-butyl-triethyl-ammonium and n-amyl-triethyl-ammonium also are powerful restoring agents. The ethyl group does not play a specific rôle in the restoration process, since restoration can also be effected by tetra-n-propyl-ammonium and tetra-n-butyl-ammonium. Restoration of nerve deprived of sodium is a function of pentavalent (tetracovalent) nitrogen.

Detailed analysis has been made in sections 9 and 10 of the participation of quaternary ammonium ions in the physiology of the nerve fiber. The analysis has led to the statement of a working hypothesis in section 12. According to the hypothesis, chemical reactions which result in a change of tricovalent nitrogen into tetracovalent nitrogen participate in the establishment of electric double layers in the nerve membrane.

The author wishes to acknowledge the assistance of Dr. A. Gallego in some experiments of a preliminary series that preceded the systematic investigation presented in this paper

LITERATURE CITED

Acheson, G. H., and G. K. Moe 1946 The action of tetraethylammonium ion on the mammalian circulation. J. Pharmacol. and Exp. Therap., *87:* 220–236.

Alles, G. A., and P. K. Knoefel 1939 Comparative physiological actions of alkyltrimethylammonium and alkali-metal salts. Univ. Cal. Publ. Pharmacol., *1:* 187–212.

Brink, F., D. W. Bronk and M. G. Larrabee 1946 Chemical excitation of nerve. Annals of the New York Academy of Sciences, *47:* 457–485.

Dale, Sir H. 1937 Transmission of nervous effects by acetylcholine. The Harvey Lectures, *32:* 228–245.

Davis, L., and R. Lorente de Nó 1947 Contribution to the mathematical theory of the electrotonus. Chapter IX, in Lorente de Nó, R., A Study of Nerve Physiology. "The Studies from The Rockefeller Institute for Medical Research." *131:* 422–446.

Erlanger, J. 1937 The analysis of the compound action potential in nerve. Chapter I, in J. Erlanger and H. S. Gasser, Electrical Signs of Nervous Activity, Philadelphia, University of Pennsylvania Press, 1–33.

Gallego, A., and R. Lorente de Nó 1947 On the effect of several monovalent ions upon frog nerve. J. Cell. and Comp. Physiol., *29:* 189–206.

Gasser, H. S. 1941 The classification of nerve fibers. Ohio J. Science, *41:* 145–159.

Guggenheim, M. 1940 Die biogenen Amine. Basel and New York, S. Karger, XVI–564.

Hofmann, A. W. 1851 Beiträge zur Kenntniss der flüchtigen organischen Basen. Ann. Chem. und Pharm., *78:* 253–286; *79:* 11 39.

Ing, H. R. 1936 The curariform action of onium salts. Physiol. Rev., *16:* 527–543.

Loevi, O. 1933 The humoral transmission of nervous impulse. The Harvey Lectures. *28:* 218–233.

Lorente de Nó, R. 1944 Effect of choline and acetylcholine chloride upon peripheral nerve fibers. J. Cell. and Comp. Physiol., *24:* 85–97.

——— 1946 Correlation of nerve activity with polarization phenomena. The Harvey Lectures, *42:* 43–105.

——— 1947 A Study of Nerve Physiology. "The Studies from The Rockefeller Institute for Medical Research." *131* and *132.*

Lorente de No, R., and T. P. Feng 1946 Analysis of the effect of barium upon nerve with particular reference to rhythmic activity. J. Cell. and Comp. Physiol., *28:* 397–464.

Overton, E. 1902 Beiträge zur allgemeinen Muskel- and Nervenphysiologie. II. Ueber die Unentbehrlichkeit von Natrium- (oder Lithium-) Ionen für den Contractionsact des Muskels. Arch. ges. Physiol., *92:* 346–386.

Raventós, J. 1937 Pharmacological actions of quaternary ammonium salts. Quart. J. Exp. Physiol., *26:* 361–374.

ON THE EFFECT OF CERTAIN QUATERNARY AMMONIUM IONS UPON FROG NERVE

Part II

By RAFAEL LORENTE DE NÓ

Twenty-Seven Figures

14. INTRODUCTION

The working hypothesis presented in section 12 has led to research along two different lines.

1. Since none of the restoring ions used in the experiments described in Part I is known to be a substance occurring in nervous tissue it seemed advisable to investigate whether or not the amino nitrogen of naturally occurring substances could be ethylated to yield a quaternary ammonium ion of the restoring type. This research has led to positive results (cf. sections 16 to 18).

2. Attempts were made to isolate from nervous tissue quaternary ammonium ions of the restoring type. This research also has led to positive results (cf. section 19). A base (or mixture of bases) has been extracted from the ox brain, which in all probability is a quaternary ammonium ion and which has the ability to restore conduction of impulses by nerve fibers deprived of sodium.

15. On the Ability of Potassium Ferrocyanide to Precipitate Quaternary Ammonium Bases

It was discovered by E. Fischer (1878) that quaternary ammonium bases are precipitated from strongly acid solution by potassium ferrocyanide. Fischer suggested the use of the reaction as a convenient method for the isolation of quaternary ammonium bases. In later years, however, the reaction has received scant mention in the literature, probably because the ability of potassium ferrocyanide to precipitate quaternary ammonium bases is not as general as Fischer believed it to be. Among others choline is a quaternary ammonium base that is not precipitated by potassium ferrocyanide.

Tests made with the 15 ions listed in figure 1 showed that potassium ferrocyanide precipitates ions I to V and IX but fails to precipitate ions VI, VII and X to XV. Ion VIII is precipitated only from concentrated solutions. Potassium ferrocyanide also precipitates the ions listed in figure 2, except ion XXV, which is not precipitated even from concentrated solutions. Thiamine (fig. 53, XXVI) is not precipitated. Ions XXVII to XXX (fig. 53) as well as ions XXXI and XXXII (fig. 58) are readily precipitated. Ions XXVII, XXVIII and XXIX, yield green ferrocyanates; all the other ferrocyanates are white.

It appears, therefore, (1) that potassium ferrocyanide fails to precipitate the three quaternary ammonium compounds that are known to exist in nervous tissue (choline, acetylcholine and thiamine), and (2) that, in addition to other ions, potassium ferrocyanide precipitates practically all the quaternary ammonium ions that are able to substitute for sodium. On the other hand, the negative results obtained with (1) the basic amino acids (arginine, histidine, lysine), (2) histamine, creatine and guanidine and (3) glycocoll betaine and the products of exhaustive ethylation of arginine and histidine, *indicate that the precipitation by potassium ferrocyanide in strongly acid medium has a high degree of specificity for quaternary ammonium ions. In particular, potassium ferrocyanide is an excellent reagent for the separation of choline from other* quaternary ammonium bases.

The precipitation by potassium ferrocyanide is done in the following manner. Sulphuric acid is added to the solution containing quaternary ammonium bases to make the solution approximately 1 to 1.5 N in H_2SO_4 and small volumes of a saturated solution of potassium ferrocyanide are added until no further precipitation occurs. The precipitate does not always form immediately; if the solution is dilute the ferrocyanate appears in crystalline form and the precipitation requires about one hour for completion. Toward the end of the precipitation it is convenient to add a few milliliters of 6 N H_2SO_4, but care must be exerted not to add a large excess of acid, because concentrated acids decompose the ferrocyanates and bring the bases again into solution.

If the precipitation is effected from a concentrated mixture coprecipitation may occur. The ferrocyanates may be purified by crystallization from hot water, as was suggested by Fischer, but since on warming a partial decomposition of the ferrocyanates always occurs, it is more convenient to recover the base and perform a second precipitation with potassium ferrocyanide from a 0.05 to 0.1 N solution.

The method suggested by Fischer for the recovery of the bases is very satisfactory. The precipitate is washed with several small volumes of cold, 0.5 or 1 N H_2SO_4 and is suspended in water. The ferrocyanide is removed with copper sulphate. With constant stirring small volumes of a 10% solution of copper sulphate are added until the filtrate begins to yield a precipitate with potassium ferrocyanide. The excess copper and the sulphate are removed from the filtrate with barium hydroxide, and the excess barium with carbon dioxide. Any amount of barium that may have escaped precipitation is removed with dilute sulphuric acid. Neutralization of the solution with hydrochloric acid yields the chloride of the quaternary ammonium base.

16. Ions of the Pyridine Group

The experiments described in this section have been done with the use of the ions listed in figure 53, XXVII to XXX.

a. Preparative technique. Ions XXVII to XXIX have been obtained by submitting pyridine, niacinamide and anhydrous coramine to the action of ethyl iodide. The niacinamide was dissolved in a small volume of water. In all cases

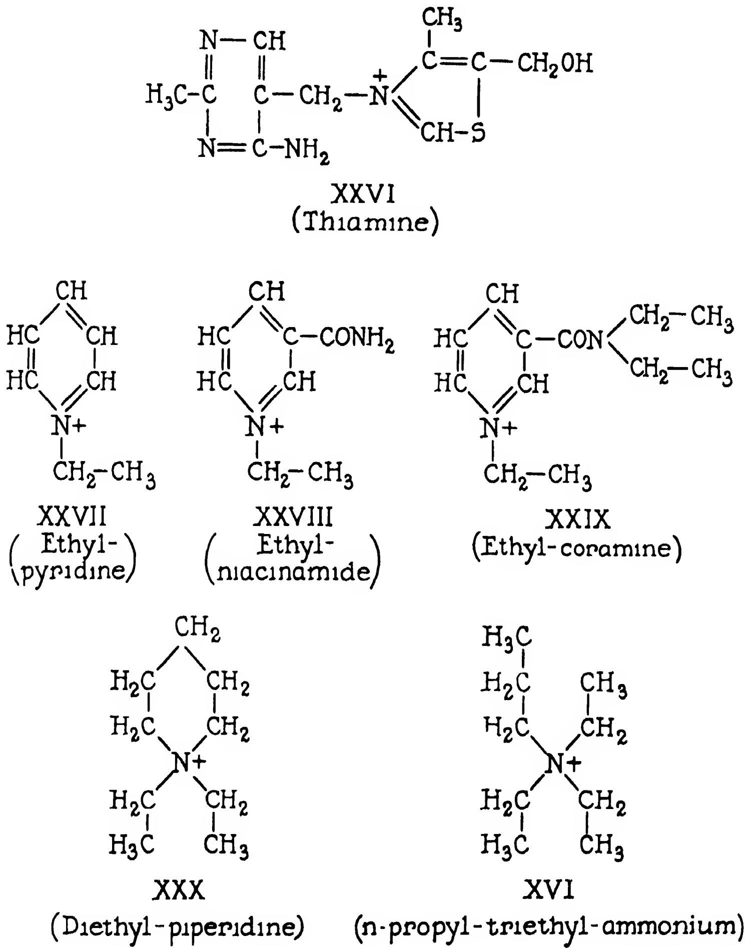


FIG. 53. Quaternary ammonium ions used in experiments described in the text.

an excess of ethyl iodide was used; the temperature of the mixture was raised at a low rate and finally, in order to insure the completeness of the reaction, the excess of ethyl iodide was allowed to boil under a reflux condenser for three to 4 hours.

The excess of ethyl iodide was removed by evaporation in a vacuum. The residue was dissolved in water and the iodide was converted into the chloride

by means of freshly precipitated silver chloride. The chlorides of the quaternary bases were obtained in crystalline form in the manner suggested by Karrer, Schwarzenbach, Benz and Solmssen ('36). The volume of the solution was reduced by evaporation in a vacuum until the solution became a thin syrup. Several volumes of absolute alcohol were added and a crystalline precipitate was obtained by the addition of several volumes of anhydrous ether.

In all cases titration of Cl^- ions in the solutions of the chlorides showed that within the accuracy of the method used (a few per cent) the amount of Cl^- ions present agreed with the theoretical molecular weight of the compound.

Ion XXX was prepared by the action of ethyl iodide upon anhydrous piperidine. The iodide was changed into the chloride by means of freshly precipitated silver chloride. The solution was made alkaline in one case by the addition of a small amount of silver oxide and in another case by the addition of a small amount of barium hydroxide. The small amount of piperidine present in the solution was removed by evaporation at 65°C., that was continued until the smell of piperidine disappeared. The barium present in one of the solutions was removed with dilute sulphuric acid. The solutions were neutralized with hydrochloric acid.

No need was felt to obtain the chloride of diethyl-piperidine in crystalline form. The concentration of the solution was adjusted by titration of Cl^- ions. Later, however, the compound was crystallized from a butyl alcohol solution.

b. Experimental results. Ions XXVII, XXVIII and XXIX are not able to restore the excitability of nerve fibers deprived of sodium, but they do not prevent later restoration by tetraethyl-ammonium. Therefore, ions XXVII to XXIX belong to the class of inert ions, i.e., at the 0.11 M concentration they maintain the osmotic equilibrium of the nerve fibers without being able to substitute for sodium and restore conduction of impulses. The effect that prolonged action of ions XXVII to XXIX could have upon the resting membrane potential has not been investigated.

Figure 54 illustrates the results of a typical experiment done with the use of ion XXVIII. The observations were begun after the central segment of the nerve (cf. fig. 3, mp_2) had performed a successful recovery in Ringer's solution. Record *1* shows that no nerve fiber was able to conduct impulses into the peripheral segment. Ethyl-niacinamide was not able to restore the excitability of any nerve fiber (records *3* to *8*), but tetraethyl-ammonium ions promptly restored the excitability of a considerable number of Et fibers (records *9* to *24*). Finally, Ringer's solution brought about an enhancement of the recovery of the Et fibers (records *25* to *32*, *34* and *35*) and effected recovery of the A fibers (records *33* and *35*).

The quaternary ammonium ion obtained by ethylation of piperidine (fig. 53, XXX) belongs to the class of restoring ions, even though its restoring ability is quite limited; on the other hand, ion XXX produces certain changes in the

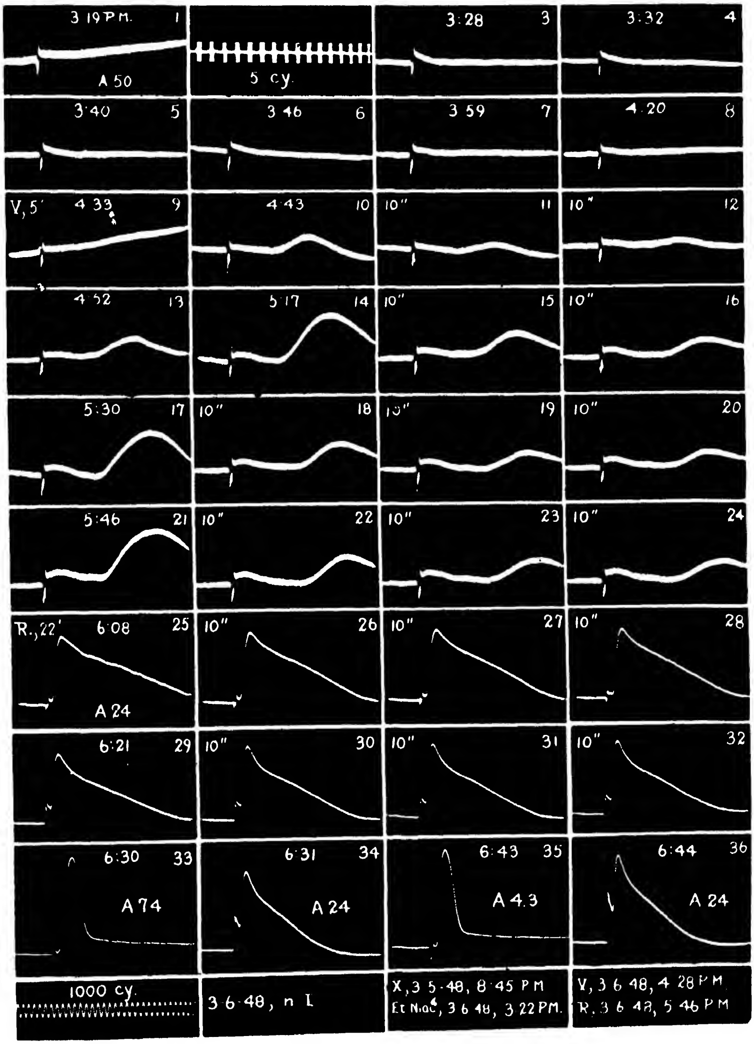


FIG. 54. Restoration of the excitability of a nerve rendered inexcitable in 0.11 M diethanol-dimethyl-ammonium chloride.

1, absence of conducted response in the peripheral segment; *3* to *8*, lack of restoration by ethyl-niacinamide; *9* to *24*, restoration by tetraethyl-ammonium; *25* to *36*, restoration by sodium. The 1000 cy time line applies to records *33* and *35*.

Et fibers which are not reversible by tetraethyl-ammonium and are reversed only with difficulty by sodium. It will be convenient to consider the situation in some detail.

The experiment illustrated by figures 55 and 56 was done with the two sciatic nerves of a bullfrog. The nerves were allowed to become inexcitable in 0.11 M diethanol-dimethyl-ammonium chloride. After the central segments had performed successful recoveries in Ringer's solution the peripheral segment of one of the nerves (n.I, fig. 55) was found to be totally inexcitable, while in the case of the other nerve (n.II, fig. 56) a small number of fibers of slow conduction still were able to conduct impulses (fig. 56, *1*).

The peripheral segment of nerve I was maintained in contact with a 0.11 M solution of the chloride of ion XXX for approximately 45 minutes. Ion XXX failed to restore the excitability of any nerve fiber (fig. 55, *3* to *6*), nor did it produce any important change in the Et fibers, since after the nerve was submitted to the action of 0.11 M tetraethyl-ammonium chloride a prompt recovery took place. A significant number of Et fibers were able to conduct impulses after 4 minutes of the action of tetraethyl-ammonium ions (record *7*), and the restored spike increased progressively in size with advancing time (records *8* to *16*). At the time when records *14* to *16* were obtained the recovery of the Et fibers was probably complete, since continued action of the restoring solution resulted only in a further increase of those changes in the spike duration and in the speed of conduction, which tetraethyl-ammonium ions are known to produce (cf. section 10). Records *21* to *23* present the conducted Et spike photographed at a smaller sweep speed.

In the case of nerve II a prompt effect of ion XXX consisted in the change of the spike that appears in record *1*,s into the spike that appears in record *3*. In all probability ion XXX caused a rapid decrease in the speed of conduction of the small group of fibers that still were excitable. The continuous growth of the conducted spike, that is shown by records *3* to *7*, proves that a later effect of ion XXX was the restoration of the ability to conduct impulses by an additional number of Et fibers. The restored spike passed through a maximum after ion XXX had acted upon the nerve for 45 minutes (records *7* to *10*); thereafter the spike height decreased continuously (records *11*, *12*). The nerve was placed in contact with 0.11 M tetraethyl-ammonium after ion XXX had acted upon it for 85 minutes; since tetraethyl-ammonium failed to improve the state of the nerve fibers it is clear that prolonged action of ion XXX had produced a change in the Et fibers, which could not be reversed by tetraethyl-ammonium. Even sodium proved to be able to restore the excitability of the nerve fibers only at an exceedingly low rate (records *17* to *27*).

Additional information is presented in figure 57. The nerve was allowed to become inexcitable in 0.11 M diethanoldimethyl-ammonium chloride and after the inexcitability had become total the peripheral segment was submitted first to the action of ion XXX and then to the action of ion V. Records *1* to *12* present the electrotonic potentials that were observed in the peripheral segment (cf. fig. 3,I). A comparison of records *2* to *4* with records *6* to *8* shows that the

polarizability of the membrane by the anodal current was decreased by the action of ion XXX; a change like this would have also occurred if the nerve had been kept in the presence of ion X, but at a lower rate. It is probable, therefore, that ion XXX exerted a slight depolarizing action upon the fibers of fast

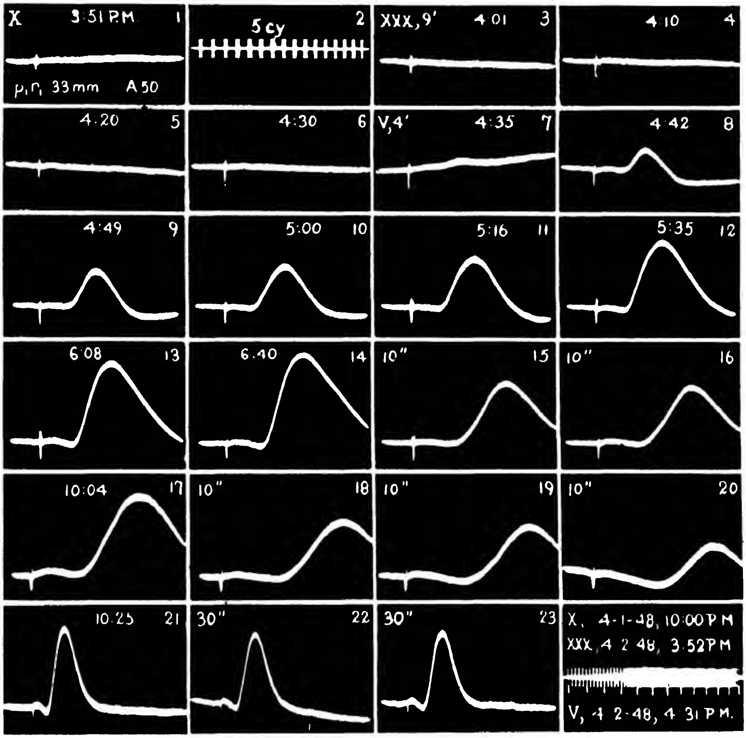


FIG. 55. Restoration of the excitability of a nerve rendered inexcitable in 0.11 M diethanol-dimethyl-ammonium chloride.

1, absence of conducted response in the peripheral segment; *3* to *6*, lack of restoration by diethyl-piperidine; *7* to *23*, restoration by tetraethyl-ammonium. Time line *2* applies to records *1* to *20*.

conduction. The effect was reversible by tetraethyl-ammonium ions, since as is shown by records *10* to *12* the polarizability of the membrane was restored by the solution of ion V; indeed, the presence of a large slow component in record *9* indicates that tetraethyl-ammonium ions were able to produce a large increase in the L fraction of the membrane potential.

In spite of the reversibility by tetraethyl-ammonium of the effect of ion XXX upon the fibers of fast conduction, the effect upon the Et fibers proved to be largely irreversible. Records *15* to *19* show that ion XXX was able to

restore the excitability only to a small number of Et fibers (records *18*, *19*, Et); it produced, however, an important change in all the Et fibers, since tetraethyl-ammonium was unable to effect a successful recovery. It is true that immedi-

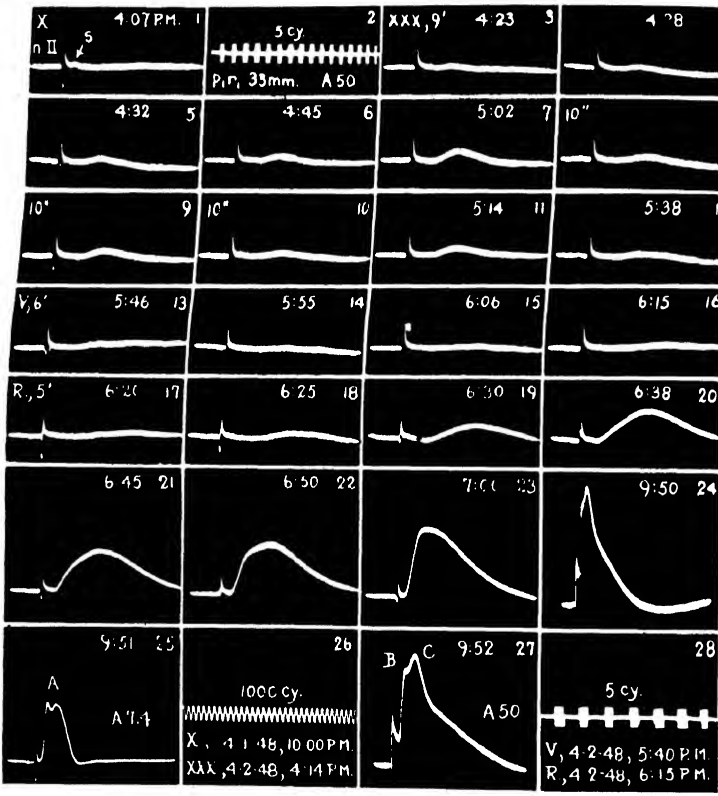


FIG. 56. From the same experiment as figure 55. *1*, s, response conducted into the peripheral segment before the application of the restoring solution; *3* to *12*, restoration by diethyl-piperidine; *13* to *16*, lack of effect of tetraethyl-ammonium; *17* to *27*, restoration by sodium. Time line *26* applies to record *25*; time line *28*, to record *27*; time line *2*, to all other records.

ately after tetraethyl-ammonium ions were made available to the nerve the conducted spike increased in size (record *20*), but thereafter the height of the spike remained practically constant (records *21* to *24*). Thus, the majority of the Et fibers had undergone a change that was not reversible by tetraethyl-ammonium.

c. Comment. The fact that ions XXVII to XXIX are unable to restore the excitability of nerve fibers deprived of sodium is in agreement with the fact

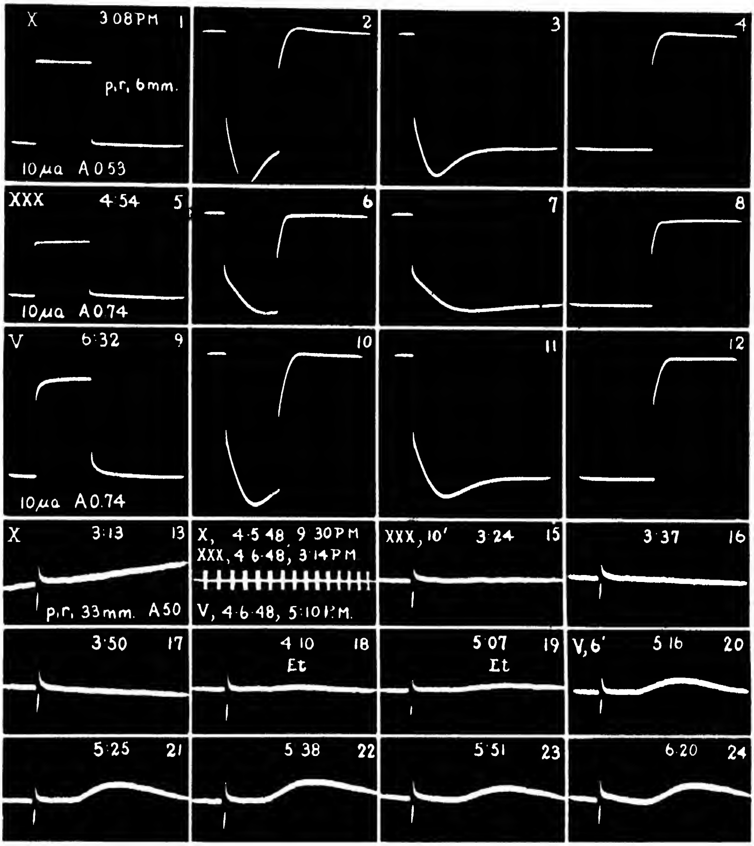


FIG. 57. Effects of diethyl-piperidine and of tetraethyl-ammonium upon nerve deprived of sodium in 0.11 M diethanol-dimethyl-ammonium chloride.

1 to *12*, electrotonic potentials in the peripheral segment; *1* to *4*, with the nerve in the presence of diethanol-dimethyl-ammonium; *5* to *8*, after the action of diethyl-piperidine; *9* to *12* after the action of tetraethyl-ammonium.

13 to *24*, tests of the ability of impulses initiated in the central segment to propagate themselves into the peripheral segment. *13*, absence of conducted response before the application of the restoring solution; *15* to *19*, effect of diethyl-piperidine; *20* to *24*, effect of tetraethyl-ammonium.

that among the ions listed in figure 1 only those which have two or more ethyl groups are restoring ions. In view of the two facts it seems justified to believe that quaternary ammonium ions in which the nitrogen belongs to a heterocyclic ring and has a double bond will not be restoring ions. This prediction is in agreement with two other facts, (1) thiamine (fig. 53) is not able to substitute

for sodium (cf. section 11) and (2) diethyl-piperidine can restore the excitability of nerve fibers deprived of sodium, even though to a limited extent.

Emphasis may be placed upon the similarities and the differences that exist between the actions upon nerve of ions III, XVI and XXX. The restoring ability of diethyl-piperidine (ion XXX) is smaller than, but comparable to that of dimethyl-diethyl-ammonium (ion III). One may be tempted, therefore, to refer the action of diethyl-piperidine to the presence of two ethyl groups in the ion, and to compare the effect of the piperidine ring with that of two methyl groups. There is, however, an important difference between the action of ions III and XXX, that suggests the assumption of an important rôle for the piperidine ring of ion XXX. As was described in section 5,c, after ion III has acted upon the nerve the restoration by tetraethyl-ammonium is only incomplete; this effect, however, is considerably weaker and develops at a lower rate than the similar effect of ion XXX. On the other hand, the effect of ion III is rapidly reversible by sodium, while that of ion XXX is reversed by sodium at an exceedingly low rate.

As was described in section 6,a ion XVI (fig. 53) has a restoring ability which is exceedingly similar to that of tetraethyl-ammonium; on the other hand, n-propyl-triethyl-ammonium exerts only a very weak depolarizing action upon the fibers of fast conduction (fig. 40, *1* and *2*). Obviously, the properties of ions XXX and XVI are essentially different, in spite of the fact that ion XXX may be regarded as a derivative of ion XVI, obtained by linking the n-propyl group and one of the ethyl groups of ion XVI to form a 5-membered ring. The reason why the introduction of a linkage like this should result in a radical change in the properties of ion XVI is not apparent; nevertheless, the fact that the change takes place is important, at least because it demonstrates clearly the dependence of the properties of quaternary ammonium ions upon links established by carbons of the groups attached to nitrogen.

17. Ion Derived from L(+) Lysine

This section presents the results of experiments done with an ion obtained by exhaustive ethylation of l(+) lysine. The results obtained are of interest for two reasons, (1) the properties of ethylated lysine are very different from those of n-hexyl-triethyl-ammonium and (2) the ε-nitrogen of lysine is generally believed to be one of the basic end groups of protein molecules.

a. Preparative technique. The procedure used for the exhaustive ethylation of l(+) lysine is essentially identical with that which was used by Engeland and Kutscher ('12) for the exhaustive methylation. Lysine dihydrochloride (Merck) was dissolved in a small amount of water and was submitted to the effect of boiling ethyl iodide under a reflux condenser. Potassium hydroxide was repeatedly added in small portions so as to keep the pH of the solution between 7 and 10. The presence in the solution of a considerable amount of a

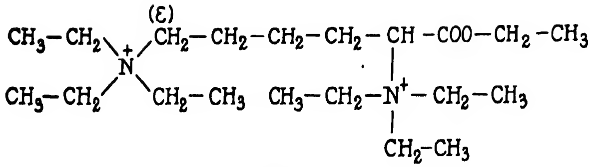
quaternary ammonium base was already detected, by a test done with the use of potassium ferrocyanide, two hours after the temperature had reached the boiling point of ethyl iodide. Nevertheless, the action of ethyl iodide was allowed to continue for 4 days, so as to insure an exhaustive ethylation of lysine. During the last two days the pH of the solution decreased at the slow rate that should be expected for the hydrolysis of ethyl iodide in a weakly alkaline medium.

The excess of ethyl iodide was removed by evaporation in a vacuum. The crystalline residue, a mixture of iodides, was dissolved in water and the iodides were converted into chlorides by the addition of freshly precipitated silver oxide, followed by neutralization with hydrochloric acid. The quaternary ammonium base was isolated as the ferrocyanate; after recovery of the base a second precipitation by potassium ferrocyanide was effected in order to insure purity of the product.

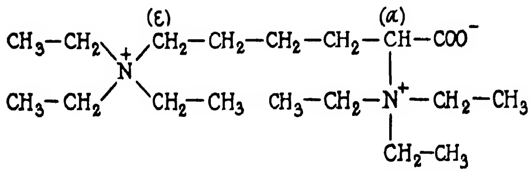
In view of the results obtained by Engeland and Kutscher ('12) it is probable that the exhaustive ethylation of lysine yielded an ethyl ester (fig. 58, XXXI,a) but it is probable that during the isolation of the base the ester was hydrolyzed so that the α-end of the lysine group acquired the betaine structure indicated in figure 58, XXXI,b. The question will be settled by the results of the analysis of the base that was used in the experiments illustrated by figures 59 and 60; for the purpose of the present discussion, however, it is sufficient to know that lysine can be ethylated to yield a strong base, that is precipitated from acid solution by potassium ferrocyanide and that is able to restore the excitability of Et fibers deprived of sodium. An important problem that is left for future investigation is to ascertain the difference between the action upon nerve of the two bases XXXIa and XXXIb. That a difference will be found is probable; the difference itself will have a great theoretical significance (see below).

b. Experimental results. Figures 59 and 60 present the results of two experiments done with the use of ethylated lysine. In one experiment (figs. 59 and 71, *1* to *16*) the effect of ethylated lysine was compared with that of brain extract 4 (fig. 71, *1* to *16* and 73, *17* to *24*); in the other experiment the effect of ethylated lysine (figs. 60 and 61) was compared with that of n-hexyl-triethyl-ammonium (figs. 62 and 63). In both experiments ethylated lysine was able to produce a far reaching recovery of the Et fibers.

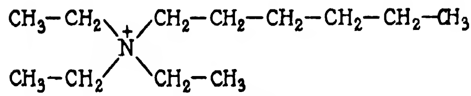
The restoration by ethylated lysine begins somewhat later than the restoration by tetraethyl-ammonium. In one case conduction by a few Et fibers was reestablished after 18 minutes (fig. 59, *4*), and in the other case after 30 minutes (fig. 60, *7*). In both cases, however, the conducted response increased progressively in size with advancing time and the restored fibers were able to conduct impulses at 10-second intervals with but little fatigue (fig. 59, *4* to *28*; fig. 60, *9* to *32*). The response remained approximately constant as long as the



XXXIa



XXXIb
(Ethyl-lysine)



XIX
(n-hexyl-triethyl-ammonium)

XXXII
(Ethyl-histamine)

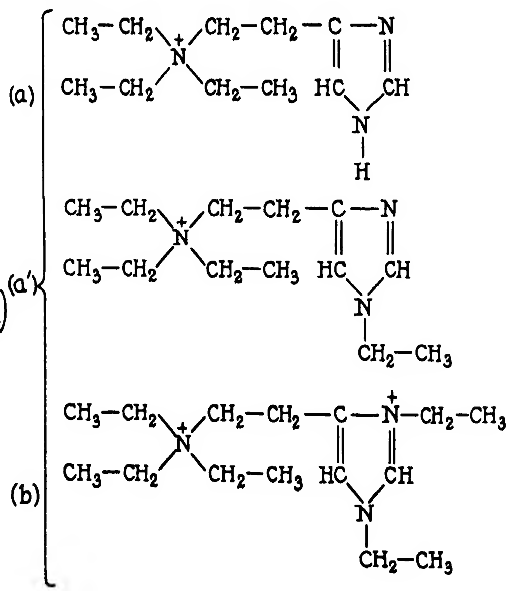


FIG. 58. Quaternary ammonium ions derived from lysine and from histamine. The formula of n-hexyl-triethyl-ammonium has been included for the purpose of comparison.

observations were continued (fig. 59, *25* to *28*; fig. 60, *29* to *32*). Records *33* and *35*, *36* of figure 60 show that after the action of ethylated lysine sodium ions were able to perform a full restoration of the nerve.

Ethylated lysine does not produce an increase in the L fraction of the membrane potential as large as that which is produced by tetraethyl-ammonium. As a matter of fact, the increase in the L fraction which is produced by ethylated

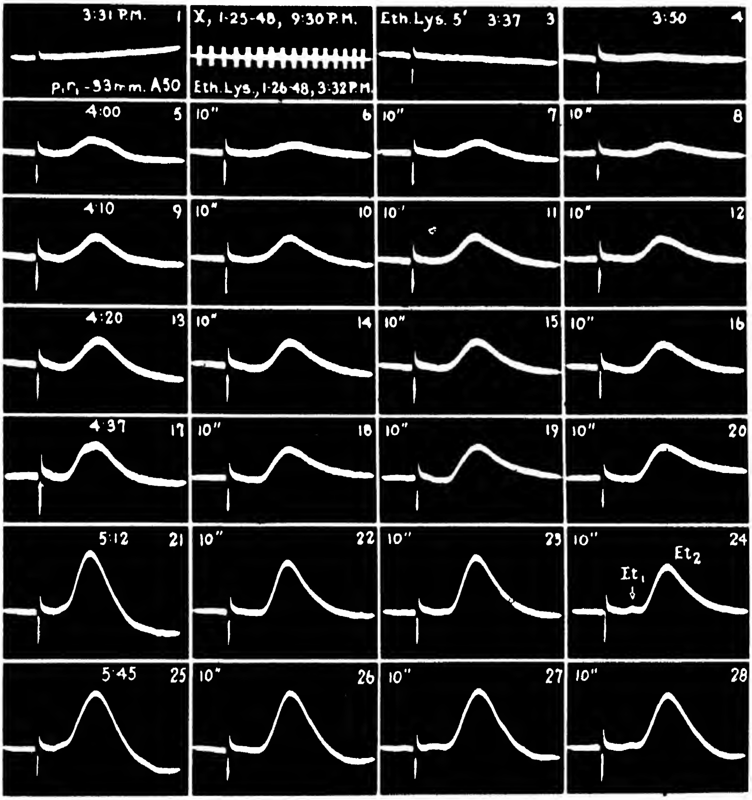


FIG. 59. Restoration by ethylated lysine of the ability to conduct impulses by Et fibers deprived of sodium.

lysine is quite small. In the experiment illustrated by figure 61, after the action of ethylated lysine had lasted for 155 minutes the slow catelectrotonus still appeared as a small deflection (records *9*, *13*); on the other hand, the slow anelectrotonus (records *10* to *12*, *14* to *16*) was only slightly greater than before (records *2* to *4*, *6* to *8*). The increase in the L fraction produced by ethylated lysine was even smaller in the experiment illustrated by figure 73, *1* to *16*.

The effect of n-hexyl-triethyl-ammonium in the experiment illustrated by figures 62 and 63, was essentially identical with the effect in the experiment

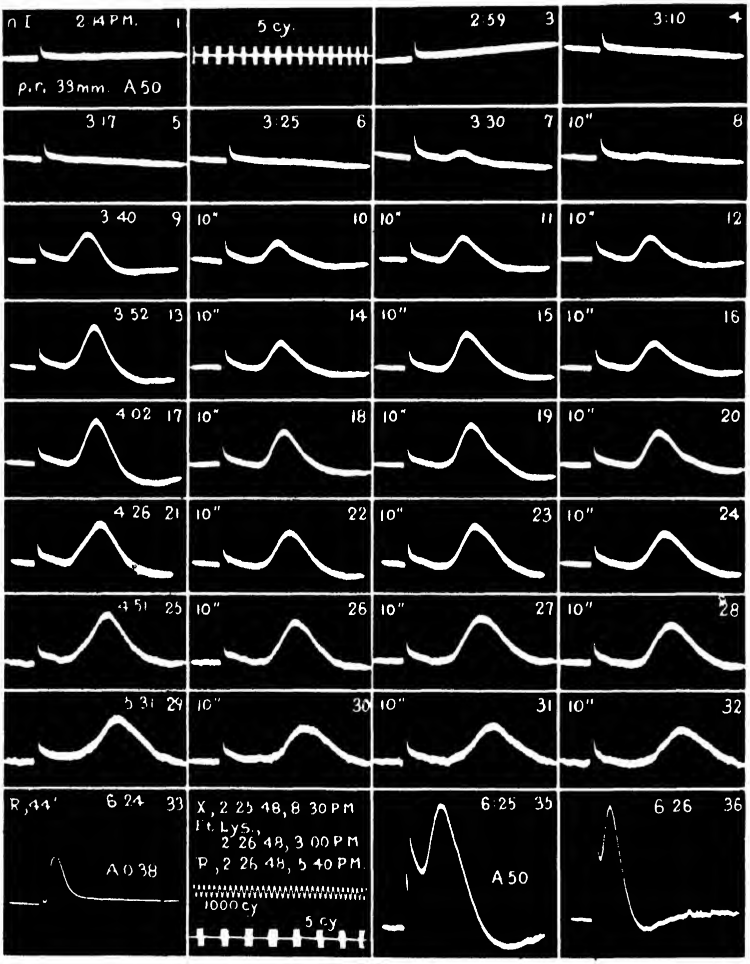


FIG. 60. Restoration by ethylated lysine (*3* to *32*) and by sodium (*33*, *35*, *36*) of the ability to conduct impulses by nerve fibers deprived of sodium. Records *1* to *32* and *36* were obtained at the same sweep speed. The 1000 cy time line in record *34* applies to record *33*, the 5 cy time line to record *35*.

reported in section 6,a (fig. 32). After the central segment of the nerve had performed a successful recovery in Ringer's solution a few fibers of slow conduction (probably C fibers) were found to be able to conduct impulses into the peripheral segment (fig. 62, *1* to *3*). No demonstrable effect of the n-hexyl-

triethyl-ammonium ions was observed during the first 20 minutes of the action of the restoring solution (fig. 62, *5* to *12*), but after 30 minutes a relatively large spike was observed (fig. 62, *13*) which was conducted at the slow speed which is so characteristic for Et fibers restored by quaternary ammonium ions. The conducted response increased in size for some time (fig. 62, *17*), but soon it began to decrease (fig. 62, *21*) and finally disappeared (fig. 62, *25*, *26*) indicating

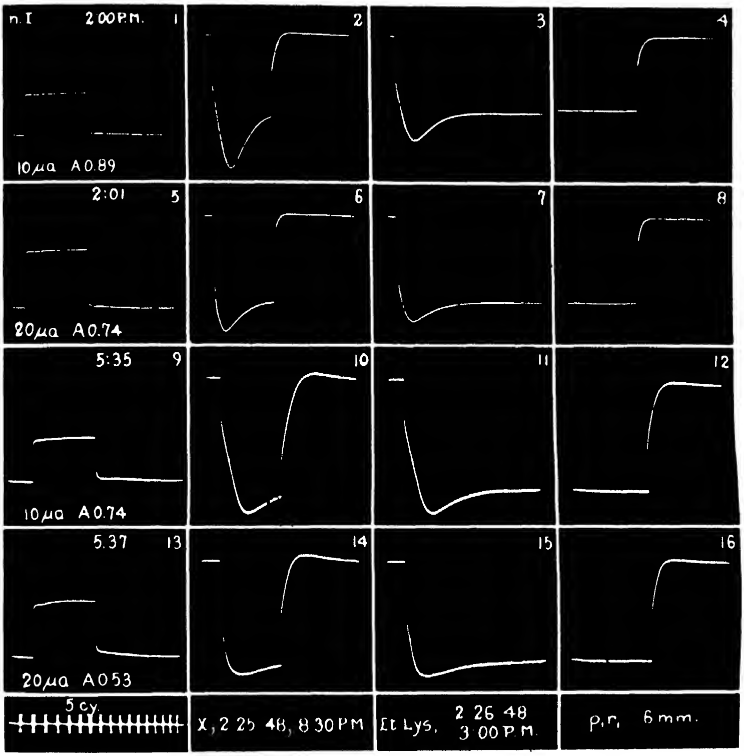


FIG. 61. Effect of ethylated lysine upon the electrotonic potentials of nerve deprived of sodium.

that n-hexyl-triethyl-ammonium had rendered all the Et fibers inexcitable. The change produced by n-hexyl-triethyl-ammonium was irreversible; Ringer's solution brought about a total restoration of the fibers of fast conduction (records *29* to *31*), but it failed to restore the excitability of any fiber of slow conduction (records *27* and *28*).

The effect of n-hexyl-triethyl-ammonium ions upon the electrotonic potentials of the fibers of fast conduction is illustrated by figure 63. A comparison of records *1* and *5* with records *9* and *13* shows that the L fraction of the membrane

potential had undergone a small, but readily detectable increase. If the differences in the amplifications used are taken into account, it will be found, by comparison of records *2* to *4* and *6* to *8* with records *10* to *12* and *14* to *16* that n-hexyl-triethyl-ammonium also had produced a slight increase in the slow

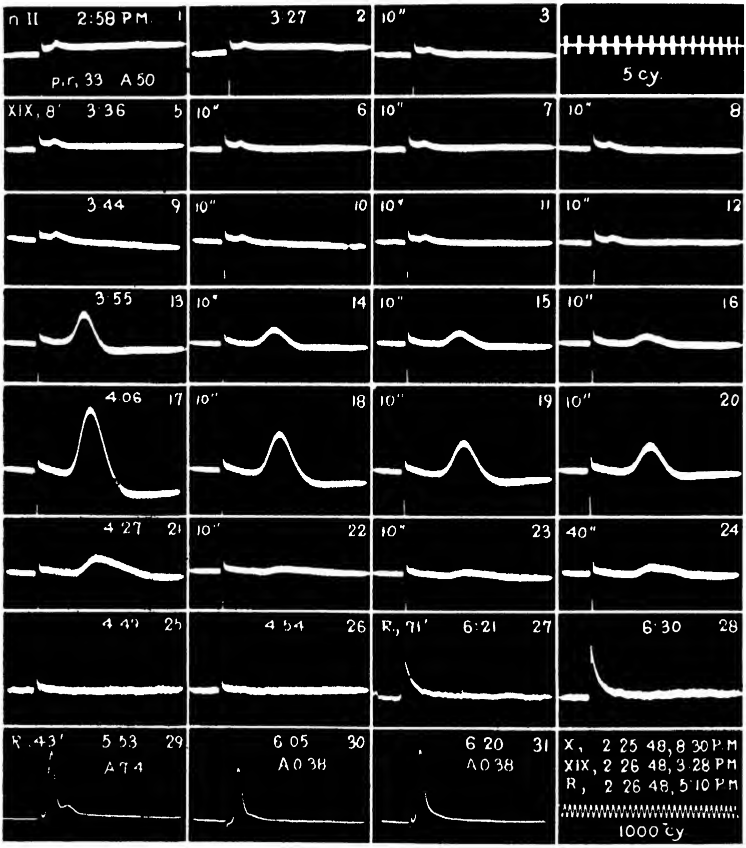


Fig. 62. Effect of n-hexyl-triethyl-ammonium (*5* to *26*) and of sodium (*27* to *31*) upon nerve fibers deprived of sodium. The 1000 cy time line applies to records *29* to *31*.

anelectrotonus. These facts indicate that although the depolarizing action of n-hexyl-triethyl-ammonium is considerable (fig. 40, *6*) the early phase of the depolarization takes place in the presence of an increased L fraction (cf. section 9,b). During this phase the effect of n-hexyl-triethyl-ammonium ions upon the fibers of fast conduction (A group) is fully reversible by sodium, while the effect upon the fibers of slow conduction (B and C groups) is already irreversible.

c. Comment. The great difference between the effects upon nerve of n-hexyl-triethyl-ammonium (fig. 58, XIX) and of ethylated lysine (fig. 58, XXXI) deserves emphasis. Lengthening of the carbon chain of the 4th group of the ions listed in column a of figure 2 produces changes in the properties of the ions, that become very important when the chain acquires 6 carbon atoms (ion XIX). If, however, the two last carbons of the n-hexyl group are replaced by

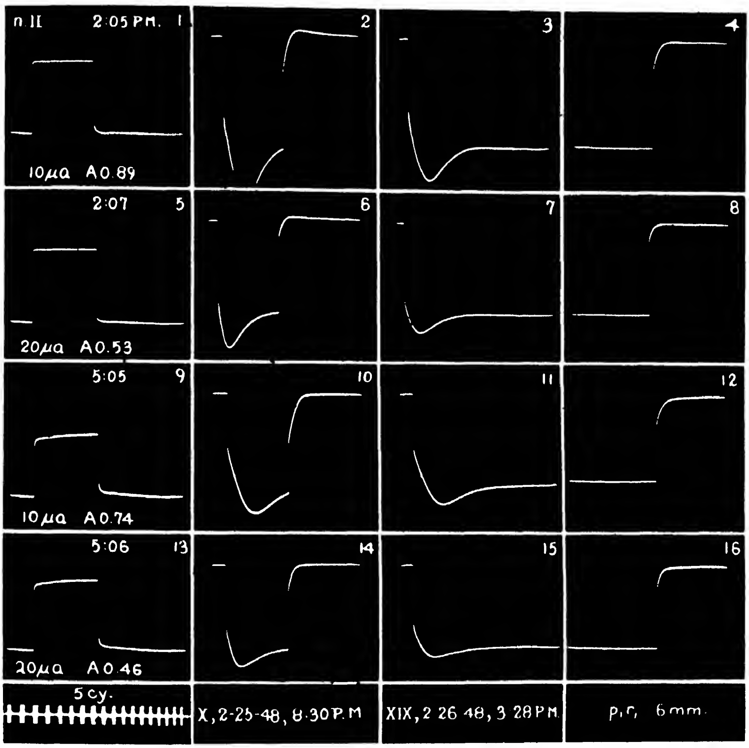


FIG. 63. Effect of n-hexyl-triethyl-ammonium upon the electrotonic potentials of nerve deprived of sodium.

one of the structures that appear in figure 58, XXXI (probably by the betaine structure XXXIb) the ion acquires properties that resemble those of ions in which the 4th group has a smaller number of carbons.

The analysis of the properties of the two ions, XXXIa and XXXIb probably will throw considerable light on the question, but, at any rate, the great difference between the properties of ethylated lysine and n-hexyl-triethyl-ammonium leaves no doubt that substitutions performed at the end of a long carbon chain may result in important changes in the properties of tetracovalent nitrogen.

The ability of ethylated lysine to restore the excitability of Et fibers deprived of sodium may be regarded as a support for the working hypothesis that was presented in section 12. The ε-nitrogen of lysine is generally believed to constitute one of the basic end groups of protein molecules. Since this nitrogen can readily be converted into a quaternary ammonium ion of the restoring type, the question may be asked whether or not the basic end groups of protein molecules are places at which amino nitrogen is converted into tetracovalent nitrogen and conversely, during the performance of nerve function. The experiments done with the use of ethylated lysine, of course, do not answer the question, but in suggesting the question they pave the way for further research. At any rate, the fact that ethylated lysine is an ion of the restoring type, proves conclusively that restoring ions can be prepared from naturally occurring substances.

18. Ions Derived from Histamine

The experiments reported in this section have been done with quaternary ammonium ions obtained by ethylation of histamine.

a. Preparative technique. In one of the preparations the ethylation of histamine was done under such conditions that the ethylation would be exhaustive. Histamine dihydrochloride (Eastman) was dissolved in a small amount of water and potassium hydroxide was added to pH 10. After the addition of an excess of ethyl iodide the temperature of the bath was raised slightly above the boiling point of ethyl iodide. The mixture was kept, of course, under a reflux condenser. Tests done after three hours with the use of potassium ferrocyanide proved the presence in the solution of a considerable amount of a quaternary ammonium base; nevertheless, the action of boiling ethyl iodide was maintained for 5 days. During this time the pH of the solution was tested at frequent intervals and whenever necessary potassium hydroxide was added to pH 9–10. After the second day the rate of decrease of the pH became practically constant indicating that the decrease was due solely to hydrolysis of ethyl iodide. Thus, in all probability the total amount of histamine was ethylated exhaustively. Since, according to the observations made by Ackermann and Kutscher ('20), exhaustive methylation of histamine results in the attachment of 5 methyl groups, it is probable that the exhaustive ethylation yielded the base XXXIIb (fig. 58).

According to the results of biological tests (see below), the ethylation of histamine yields a different quaternary ammonium base if the pH of the mixture is never brought above 8 and is maintained well below 7 during most of the time. Histamine dihydrochloride was dissolved in a small amount of water and the solution was brought to pH 7 by the addition of potassium hydroxide. An excess of ethyl iodide was added and the temperature of the bath was raised slightly above the boiling point of ethyl iodide. The presence

of a quaternary ammonium base in the solution soon became detectable; since the solution also had become strongly acid, potassium hydroxide was added to neutralize the hydroiodic acid. The addition of potassium hydroxide was repeated at infrequent intervals in amounts such that the pH of the solution never became higher than 8. The action of boiling ethyl iodide was maintained for 5 days, but no potassium hydroxide was added after the 4th day, so that during the last 24 hours a considerable amount of hydroiodic acid was present in the mixture.

The exact formula of the quaternary ammonium base that was obtained is not known. In view of the fact that the product of exhaustive ethylation of histidine is not precipitated by potassium ferrocyanide, and also for convincing theoretical reasons, it may be taken for granted that the quaternary ammonium base had resulted from the attachment of three ethyl groups to the amino nitrogen of histamine. Thus, the difference between the two bases that have been derived from histamine can consist only in the number of ethyl groups attached to the imidazol ring. Probably, the base obtained by ethylation of histamine in acid medium is one of the bases XXXIIa or XXXIIa′. For the purposes of the present discussion it is not essential to know the exact formulae of the histamine derivatives that have been used. The important point is that, in all probability, the bases differ only in the number of ethyl groups attached to the imidazol ring, as is suggested by formulae XXXIIa, XXXIIa′ and XXXIIb. For convenience, the base obtained by ethylation of histamine in acid medium will be called base a, and the base obtained by ethylation in basic medium, base b.

The isolation of the bases was done in the following manner. After removal of the excess of ethyl iodide by evaporation in a vacuum the residue was dissolved in a small volume of ethyl alcohol to remove part of the potassium iodide. The dissolved iodides were converted into chlorides by means of an excess of lead chloride. The quaternary ammonium bases were then isolated as ferrocyanates. After recovery of the bases a second precipitation was effected with potassium ferrocyanide. Although the chlorides of the two bases are hygroscopic they can readily be crystallized from aqueous solution by slow evaporation in a dry atmosphere. They also can be obtained as crystalline precipitates from alcohol solution by the addition of ether.

The chloride of base a, has been used only at one concentration, 0.11 N in Cl^- ions; the chloride of base b, at two concentrations, 0.11 N in Cl^- ions (fig. 64) and 0.14 N in Cl^- ions (figs. 65 to 68 and 70). That the difference in the concentrations would fail to produce a detectable change in the experimental results was fully expected, since even relatively large changes in the osmotic pressure of the external medium have very little effect upon frog nerve (cf. section 4,a).

b. Experimental results. The base (b) resulting from the exhaustive ethylation

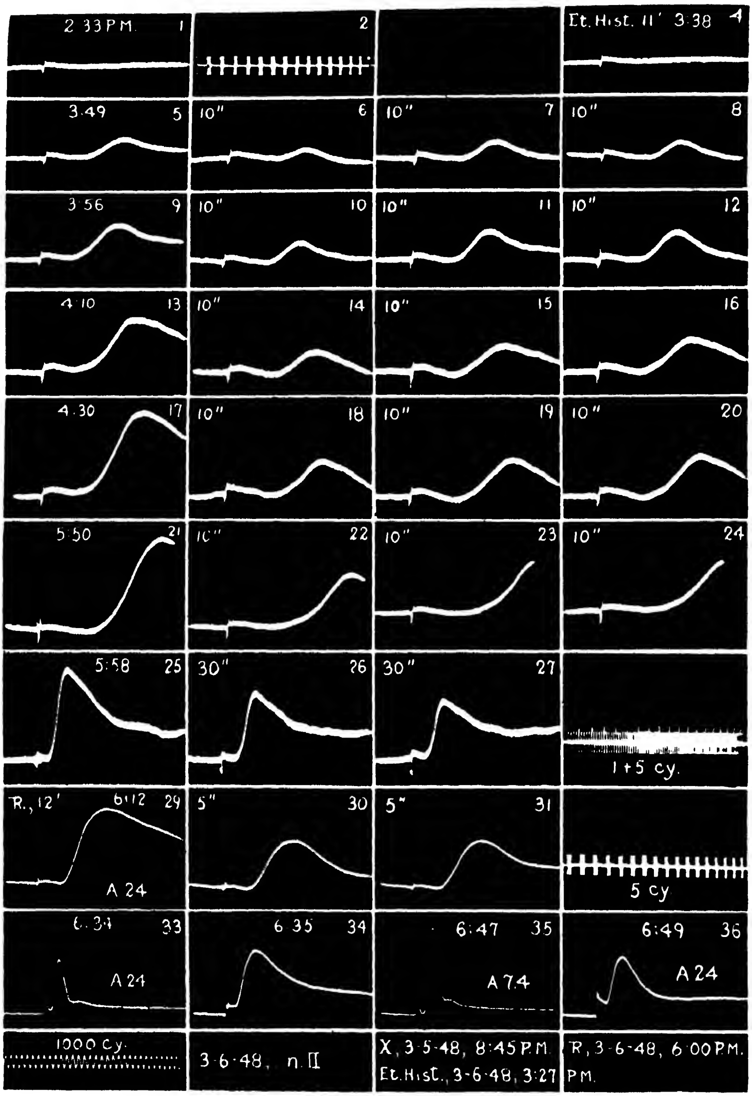


FIG. 64. Restoration of the excitability of the peripheral segment of a nerve rendered inexcitable in 0.11 M diethanol-dimethyl-ammonium chloride.

1, absence of conducted response in the peripheral segment of the nerve; *4* to *27*, restoration of the excitability of Et fibers by ethylated histamine (base b); the amplification for records *1* to *27* was constant (A 50). Records *1* to *24* were obtained with the sweep speed of record *2*; records *25* to *27*, with that of record *28*. *29* to *36*, restoration by Ringer's solution. Time line *32* applies to records *29* to *31*, *34* and *36*; the time line below record *33*, to records *33* and *35*.

of histamine in a basic medium has an action upon frog nerve deprived of sodium which is remarkably similar to that of tetraethyl-ammonium.

In the experiment illustrated by figure 64 the nerve was allowed to become inexcitable in a 0.11 M solution of diethanol-dimethyl-ammonium chloride. After the central segment had performed a successful recovery in Ringer's solution the peripheral segment was found to be totally inexcitable (fig. 64, *1*). No conducted response was observed after the action of ethylated histamine had lasted for 11 minutes (record *4*), but 11 minutes later a considerable number of Et fibers were able to conduct impulses (records *5* to *8*). The conducted response increased progressively in size as long as the observations were continued (records *9* to *24*). Up to the time when record *17* was obtained the increase in the response must have been produced chiefly by an increase in the number of conducting fibers, but thereafter the increase must have been caused by an increase in the height of the individual fiber spikes. From a comparison of records *17* and *21* it appears that the increase in the spike height was accompanied by a decrease in the speed of conduction and by a lengthening of the spike duration. Records *25* to *27*, that were obtained at a lower sweep speed, show the great duration of the spike, that was caused chiefly by the great height and low rate of decay of the negative after-potential. Thus, ethylated histamine was producing those changes in the properties of the Et fibers, which tetraethyl-ammonium is known to produce; the only difference consisted in that the effect of ethylated histamine was even stronger than that of tetraethyl-ammonium.

The changes produced by ethylated histamine were reversed by sodium. After 12 minutes of the action of sodium ions the Et spike still had a great height and a long duration, it also was conducted at a very low speed; with advancing time, however, the height and the duration of the Et spike decreased progressively and the speed of conduction increased (records *34* and *36*). Sodium also restored the excitability of the fibers of fast conduction (fig. 64, *33*, *35*).

The effect of base b upon the electrotonic potential is illustrated by figure 65. Since at the time when the observations were begun the polarizability of the membrane by the anodal current still was high (records *2* to *4*) the effect of ethylated histamine upon the anelectrotonus revealed itself only in an increase in the effectiveness of the nerve reaction (records *6* to *8* and *10* to *12*). In the case of the catelectrotonus, however, ethylated histamine produced a spectacular increase in the height of the slow component (records *1*, *5*, *9*) which was a measure of the increase in the L fraction of the membrane potential.

Records *13* to *15* that were obtained at a higher sweep speed illustrate an interesting effect of the increase in the value of the L fraction. Except for the presence of an E_1 deflection at the make of the current, record *13* presents the catelectrotonus almost as a rectangular deflection, while after the action

of ethylated histamine the growth and the decay of the slow catelectrotonus became the most prominent features of the records. The growth and the decay

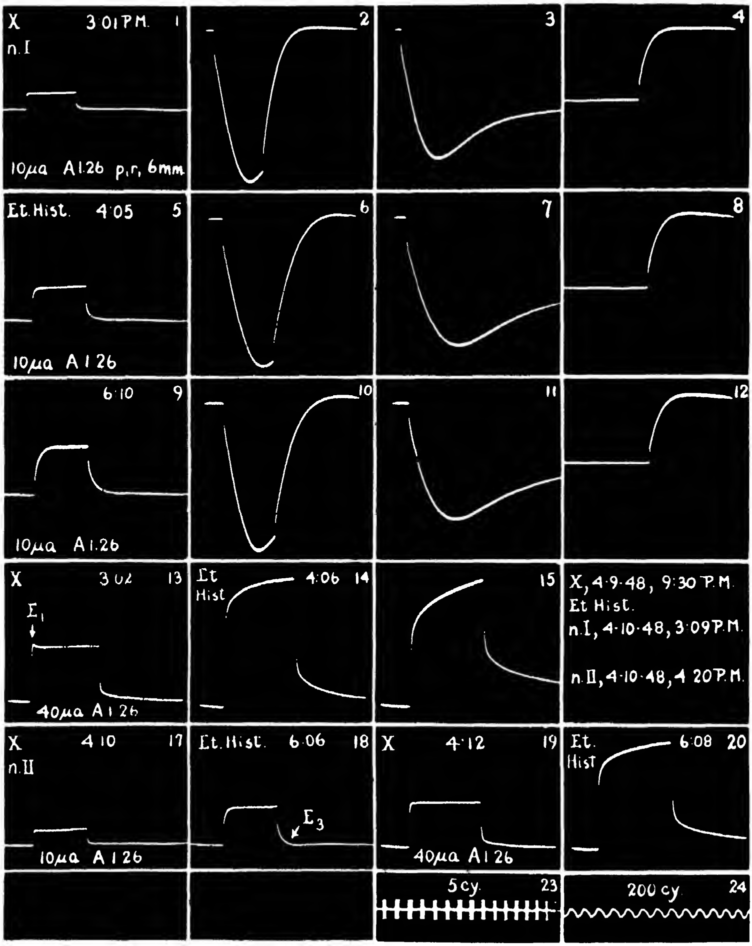


FIG. 65. Effect of ethylated histamine (base b) upon the electrotonic potentials of nerve deprived of sodium. Time line *23* applies to records *1* to *12* and *17*, *18*; time line *24*, to records *13* to *15* and *19*, *20*. Record *15* was obtained at 6:12 P.M.

of the slow catelectrotonus were slower in the case of record *15* than in that of record *14* because during the intervening time the L fraction of the membrane potential had undergone an important increase (cf. records *5* and *9*).

Records *17* to *20* present the changes in the catelectrotonus that were observed with nerve II. Since this nerve was left in diethanol-dimethyl-am-

monium chloride for an additional length of time, no E_1 deflection is present in record *19*.

Figure 66 shows that the action of base a upon the nerve is different from that of base b. Consideration of this experiment will also serve to emphasize the differences that exist between the A and the Et fibers. At the time when the observations were begun a few fibers of the A group (record *1*) as well as a few fibers of the C group (record *2*) were able to conduct impulses into the peripheral

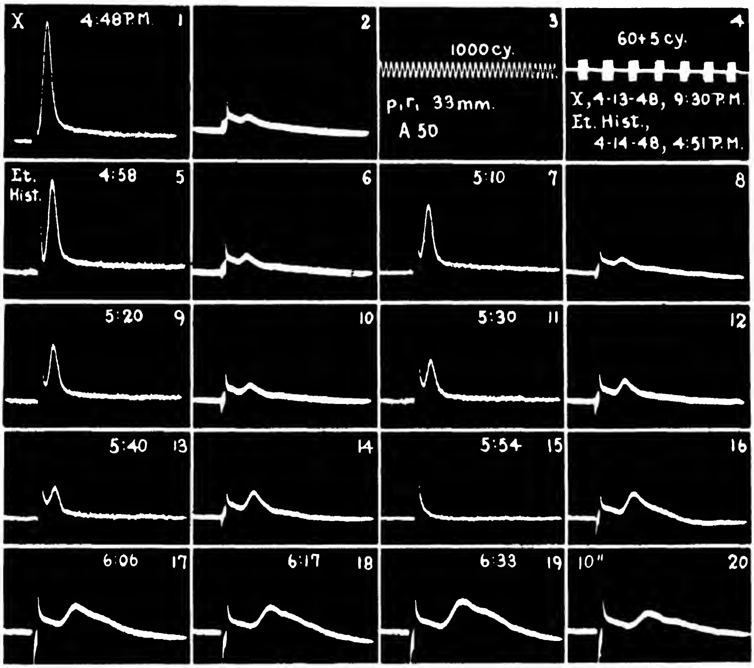


FIG. 66. Effect of ethylated histamine (base a) upon the excitability of nerve fibers deprived of sodium. Time line *3* applies to records *1*, *5*, *7*, *11*, *13* and *15*; time line *4*, to all the other records.

segment. In the presence of ethylated histamine (base a) the A fibers progressively lost their ability to conduct impulses (records *5*, *7*, *9*, *11*, *13*, *15*), probably in the same manner in which they would have become inexcitable in any inert sodium-free medium. In contrast with its lack of action upon the A fibers, ethylated histamine kept excitable those fibers of slow conduction which still were able to conduct impulses, and in addition restored the excitability of a number of those fibers which already had become inexcitable (records *6*, *8*, *10*, *12*, *14* and *16* to *20*). The only remarkable thing was that base a restored the excitability of only a small number of fibers.

A detail that deserves mention is this. During the early phases of the restoration the impulses were conducted at approximately the same speed as before the application of the restoring solution (cf. record *2* with records *6*, *8*, *10*, *12*), but with continuing action of the restoring solution the speed of conduction decreased progressively (records *16* to *20*). This observation is an example of a general rule: if the excitability of Et fibers is restored by quaternary ammonium ions immediately after the fibers have become inexcitable, the speed of conduction of the impulses is greater than it would be if the restoration had been effected after the nerve fibers have been inexcitable for some time. Another example of this rule is figure 15, *17* to *24* (cf. fig. 19, *5* to *28*). If the action of the restoring ion is allowed to continue, the speed of conduction decreases progressively, as is shown by the records reproduced in figure 66.

A drastic demonstration of the difference between the actions of the two bases derived from histamine is presented in figure 67. The experiment was done with the two sciatic nerves of a bullfrog. The nerves were allowed to become inexcitable in 0.11 M diethanol-dimethyl-ammonium chloride. In the case of nerve I the restoration of the peripheral segment was effected with preparation b of ethylated histamine (fig. 67, *1* to *20*), and in the case of nerve II with preparation a (fig. 67, *21* to *35*). Base b rapidly restored the ability to conduct impulses to a large number of Et fibers (records *3*, *4*) and the conducted response increased continuously in size, as long as the observations were continued (records *5* to *20*). Base a acted at a much lower rate, since a conducted response was not observed until after the nerve had been in contact with the restoring solution for 15 minutes (record *25*). The conducted response increased but little during continued action of base a (records *26* to *32*); indeed, the response soon began to decrease (records *33* to *35*), to disappear altogether (record *36*).

A direct comparison of the two bases was again made in the experiment illustrated by figures 68 to 70. Consideration of this experiment will also serve to indicate the extent to which differences between individual nerves may influence the experimental results. Indeed, the experiment illustrated by figures 68 to 70 will serve to show that unless the available experimental material is extensive one might easily be led to erroneous conclusions. If one compares records *1* to *20* of figure 67 with figure 69, one would conclude that bases a and b have approximately equal actions upon nerve; but if one compares the results obtained with the two nerves of each pair, i.e., records *1* to *20* of figure 67 with records *21* to *36* of the same figure and figure 69 with figure 70, one cannot fail to realize that the actions upon nerve of bases a and b are quite different. The differences between individual nerves could hardly be explained in any other manner than in terms of metabolic conditions in the body of the animal at the time of excision of the nerves.

The experiment illustrated by figures 68 to 70 were done with the two

sciatic nerves of a bullfrog. The nerves were allowed to become inexcitable in 0.11 M diethanol-dimethyl-ammonium. Figure 58, *1* to *4* and *13* to *16* presents the electrotonic potentials recorded in the peripheral segments of the two nerves

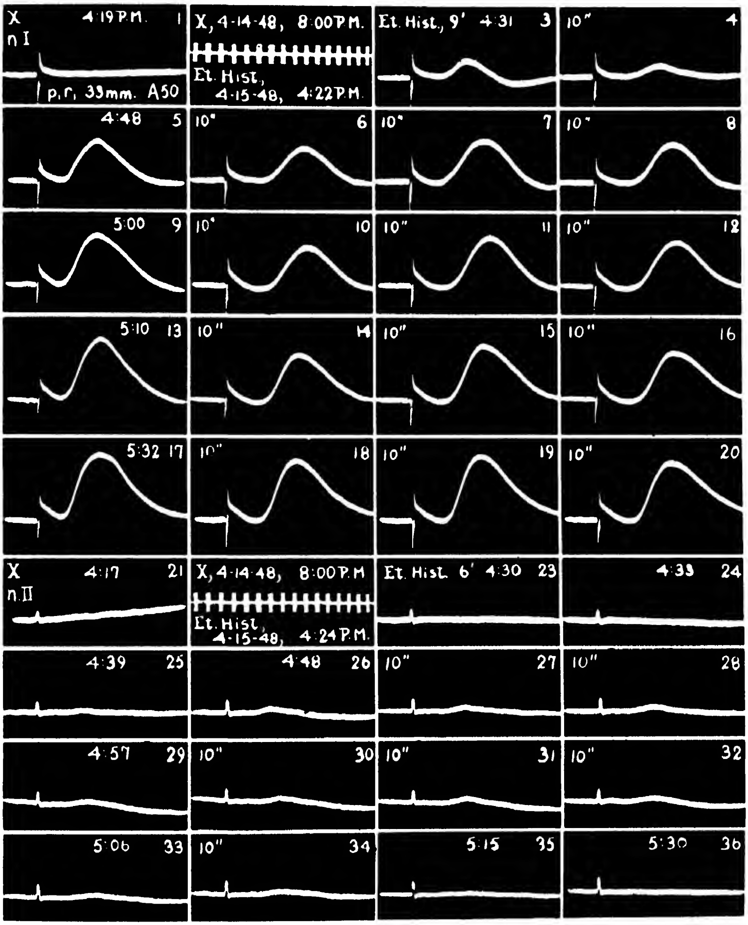


Fig. 67. Effect of the two quaternary ammonium bases derived from histamine upon the excitability of nerve fibers deprived of sodium. *1* to *20*, effect of base b; *21* to *36*, effect of base a.

before the application of the restoring solutions; as can readily be seen, the states of the two nerves were practically identical. Preparation a of ethylated histamine produced only a small increase in the L fraction of the membrane potential, as is proven by the small height of the slow catelectrotonus in records *5* and *9*; it also produced only a small increase in the polarizability of the mem-

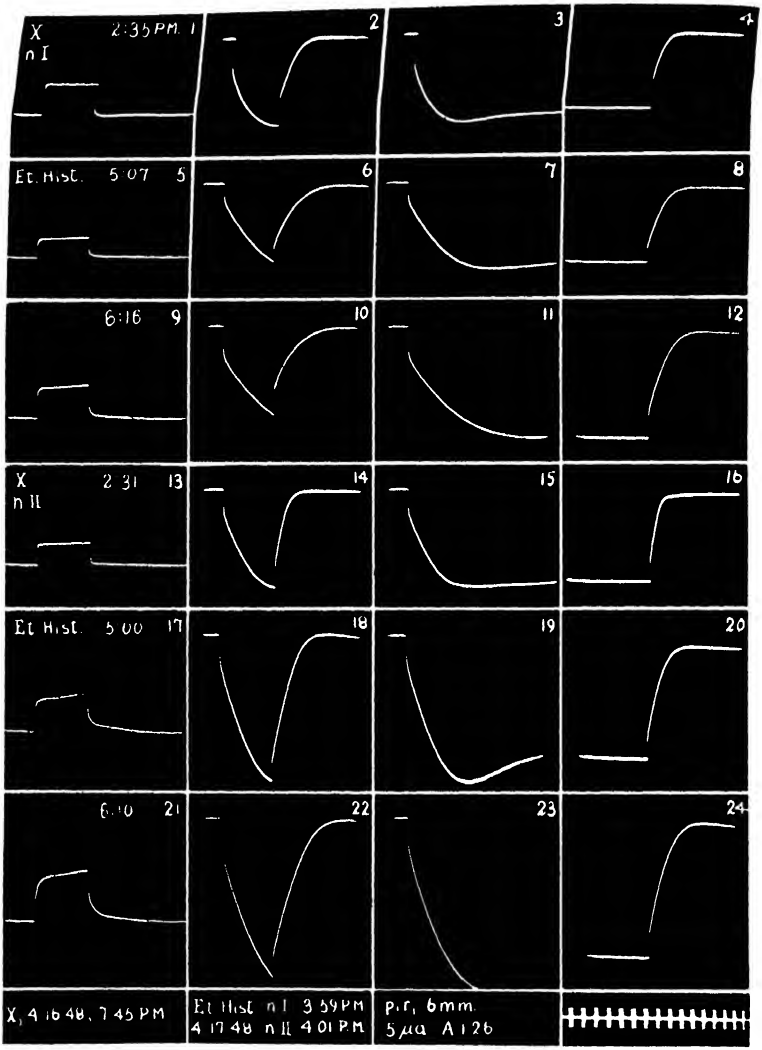


FIG. 68. Effect of the two quaternary ammonium bases derived from histamine upon the electrotonic potentials of nerve deprived of sodium. *1* to *12*, effect of base a; *13* to *24*, effect of base b.

brane by the anodal current (cf. records *2* to *4*, *6* to *8*, and *10* to *12*). Preparation b of ethylated histamine produced a large increase in the L fraction, that is measured by the height of the slow electrotonus in records *17* to *21*; it also produced a large increase in the polarizability of the membrane by the anodal current (records *14* to *16*, *18* to *20*, *22* to *24*).

Figures 69 and 70 illustrate the recovery by Et fibers of the ability to conduct impulses. Base a (fig. 69) initiated the recovery later than base b (fig. 70). The response restored by base a increased progressively in size to reach a maximum in about 90 minutes (fig. 69, *6* to *20*); thereafter it decreased slightly

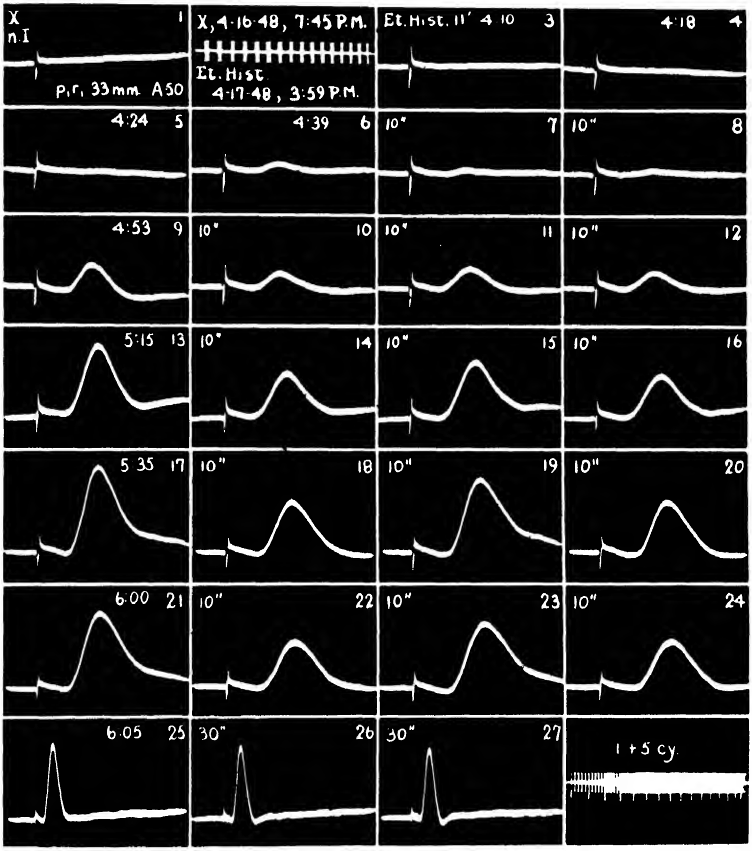


FIG. 69. Effect of ethylated histamine (base a) upon the excitability of nerve deprived of sodium. Time line *2* applies to records *1* to *24*.

(records *21* to *24*). The response restored by base b increased continuously as long as the observations were continued (fig. 70, *7* to *24*); the increase in height of the conducted response which was observed during the early part of the experiment (fig. 70, *7* to *12*) undoubtedly was due in the main to an increase in the number of conducting fibers; the later increase (fig. 70, *13* to *24*), that was accompanied by a great lengthening of the spike duration, must have been due to an increase in the height of the individual fiber spikes. The contrast

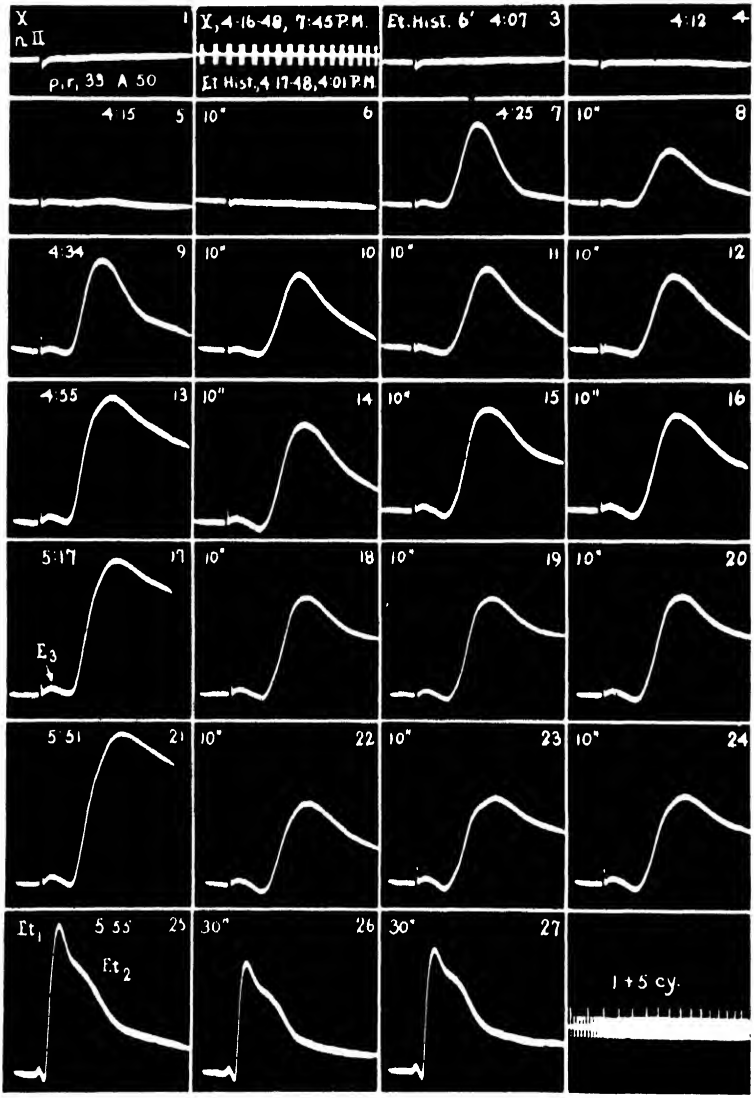


FIG. 70. Effect of ethylated histamine (base b) upon the excitability of nerve deprived of sodium. Time line *2* applies to records *1* to *24*. The deflection labelled E_3 in record *17* is the electrotonic potential produced in A fibers by the stimulating current.

between the spikes restored by the two bases was a drastic proof of the difference between the actions of bases a and b. The contrast appeared with particular

clarity when the observations were repeated at a slower sweep speed. The spike restored by base a (fig. 69, *25* to *27*) had, of course, an abnormally long duration, but this duration was only a fraction of that of the spike restored by base b (fig. 70, *25* to *27*). As a matter of fact, the effect of base b upon the spike duration of Et fibers is greater than the effect that is produced by any other quaternary ammonium ion in a comparable length of time. It is only when tetraethyl-ammonium is allowed to act upon the nerve for an extended period of time that the Et spike (fig. 45, *13*, *17*, *23*, *24*) becomes as long as the spikes reproduced in figure 70, *21* and *25*. To a large extent the great duration of the spike is referable to the slow decay of the negative after-potential, i.e., to the slow recovery of an abnormally great L fraction of the membrane potential.

c. Comment. That ethylated histamine has properties different from those of histamine itself is not a new fact. Ackermann and Kutscher ('20) and Vartiainen ('35) have shown that the quaternary ammonium base resulting from the methylation of histamine has properties different from those of the parent substance. In the case of histamine the properties of the substance are due chiefly to the presence of the imidazol ring; in the case of ethylated histamine the properties of the substance are chiefly those of a quaternary ammonium ion with three ethyl groups. The presence of the imidazol ring, however, still plays an important rôle, as is shown by the fact that preparations a and b have different actions upon nerve.

No overemphasis can be placed upon this difference. The exact formulae of bases a and b are not known, but there can be hardly any doubt that the two bases differ only in the number of ethyl groups attached to the imidazol ring. That bases so similar in structure act upon nerve in unequal manners is a further example of the close dependence of the properties of tetracovalent nitrogen upon details of structure of the groups attached to it. Since a large number of variations can be introduced in the imidazol ring it may be expected that systematic study of quaternary ammonium bases with three ethyl groups derived from substituted histamines will throw considerable light on the problem.

19. Effect upon Nerve Deprived of Sodium of a Base (or Mixture of Bases) Extracted from Ox Brain

The experiments described in this section have been done with the use of substances extracted from the ox brain, in an attempt to answer the question whether or not nervous tissue contains restoring quaternary ammonium ions.

a. Method of extraction. The literature contains excellent presentations of the methods that have been used to isolate basic substances from brain, muscle and other tissues. Particularly instructive are the descriptions made by Barger ('14), Guggenheim ('23 and '40) and Winterstein ('33). After careful con-

sideration of the advantages and disadvantages of the various methods it was decided that the most promising method would be this: To prepare an acid hydrolysate of brain tissue and to submit the hydrolysate to the various steps of the classical Kossel-Kutscher procedure for the isolation of the basic amino acids. The so-called "lysine fraction" would contain, in addition to choline, the quaternary ammonium ions of the restoring type, if such ions were present in the brain. Precipitation by potassium ferrocyanide would serve to isolate the quaternary ammonium ions of the restoring type (cf. section 15).

Three small scale extracts were prepared in order to work out details of procedure. The crude lysine fractions of extracts 2 and 3 proved to contain basic substances having a definite action upon nerve. Consequently, it was decided to undertake a new extraction on a somewhat larger scale. It will be convenient to describe in some detail the preparation of brain extract 4.

Ox brains (5 kg) were minced in 5 l of distilled water and concentrated hydrochloric acid was added to make the mixture approximately 0.1 N in HCl. The mixture was boiled under a reflux condenser for 4 hours and the volume of the mixture was reduced to 4 l by boiling at atmospheric pressure. The filtrate was neutralized with barium hydroxide and basic lead acetate was added until no further precipitation occurred. Sulphuric acid was added to the filtrate to pH 5 and the filtrate was concentrated on the water bath (approximately 65°C.) to a volume of 2 l. A second precipitation was performed with basic lead acetate and the excess lead was removed with hydrogen sulphide.

The filtrate was concentrated to 2 l by evaporation on the water bath. The basic substances were precipitated with 20% phosphotungstic acid in the presence of 5% sulphuric acid. The precipitate was washed several times with 5% sulphuric acid and finally with distilled water. The bases were recovered by means of barium hydroxide and the excess barium was removed with carbon dioxide.

Sulphuric acid was added to the filtrate to pH 5 and the volume of the solution was reduced to 1 l on the water bath. A boiling saturated solution of silver sulphate was added until Kossel-Kutscher's "brown spot" test became positive. The precipitate was discarded. To the filtrate finely pulverized barium hydroxide and then a saturated solution of barium hydroxide was added to pH 9. The abundant precipitate was removed by centrifugation and discarded. The excess barium was removed from the supernatant by means of carbon dioxide and hydrogen sulphide was passed through the solution to remove any silver that might have escaped precipitation.

The filtrate was concentrated on the water bath to approximately 200 ml and sulphuric acid was added to make the solution approximately 1 N in H_2SO_4. Small volumes of a saturated solution of potassium ferrocyanide were added until no further precipitation occurred. The filtrate was discarded. From the precipitate the basic substances were recovered by means of copper

sulphate and barium hydroxide in the manner described in section 15. Neutralization with hydrochloric acid yielded a solution of the chlorides of the bases.

The concentration of the solution was adjusted to 0.1 N in Cl^- ions; the solution proved to contain a base that has the ability to restore the excitability of nerve fibers deprived of sodium (fig. 71, *1* to *16*), but since the solution had an orange color it was decided to attempt a purification of the extract. A second precipitation with potassium ferrocyanide was not deemed advisable, because it would cause a loss of active substance during the washing of the ferrocyanate. The solution was acidified with hydrochloric acid to pH 4.5 and evaporated to dryness on the water bath. The residue was extracted with a small volume of boiling methyl alcohol; a part of the residue failed to dissolve, but later tests (fig. 71, *17* to *31*) proved that the activity of the brain extract had not decreased. The methyl alcohol solution was evaporated and the residue dried in a vacuum at 80°C. The residue consisted in part of a crystalline powder, probably white, and in part of an amorphous greenish-yellow material. Both matters were very soluble in water, readily soluble in methyl alcohol and almost insoluble in cold ethyl alcohol. No attempt was made to separate the two matters. Finally, the residue was dissolved in a small amount of water.[12]

The solution was practically neutral (pH 6.7); therefore, it contained the chloride of a strong base (or bases). For its use on experiments on nerve the volume of the solution was adjusted so that the concentration of Cl^- ions became 0.1 N. The volume of the solution was then 21 ml; thus, on the assumption that the active substance is a monoacid base, the yield of the extraction from 5 kg of brains was approximately 2 millimoles. Appropriate tests showed that the solution contained only traces of potassium and of sodium, i.e., that these two ions were present only in amounts many times smaller than those which still have demonstrable effect upon frog nerve.

b. Experimental results. Figure 71 presents records, obtained in two different experiments, that illustrate the effect of brain extract 4 upon the fibers of slow conduction of frog nerve deprived of sodium. One of the experiments (records *1* to *16*) was done before the extract had been purified; the other (records *17* to *31*), after the removal of impurities insoluble in methyl alcohol The experiments were done in the usual manner. The nerves were allowed to become inexcitable in a large volume of 0.11 M diethanol-dimethyl-ammonium chloride and after the nerves had been mounted in the moist chambers the central segments were allowed to perform their recoveries in Ringer's solution.

[12] After the performance of the experiments described in text, ethyl alcohol was added to extract 4 until the solution became slightly turbid. The solution has been kept in a dessicator over calcium oxide. As a result of the slow removal of water two matters are separating again: a small amount of a white powder, chiefly on the walls of the vessel, and a much greater amount of a red matter in the form of numerous globules of rather uniform size, chiefly on the bottom of the vessel.

As is shown by records *1* and *17* no nerve fiber was able to conduct impulses into the peripheral segment of the nerves. The brain extract restored the ability to conduct impulses to a number of fibers of slow conduction. In one case (records *5, 6*) a conducted response was observed after the extract had acted upon the nerve for 25 minutes, and in the other case (record *21*) after 29 minutes.

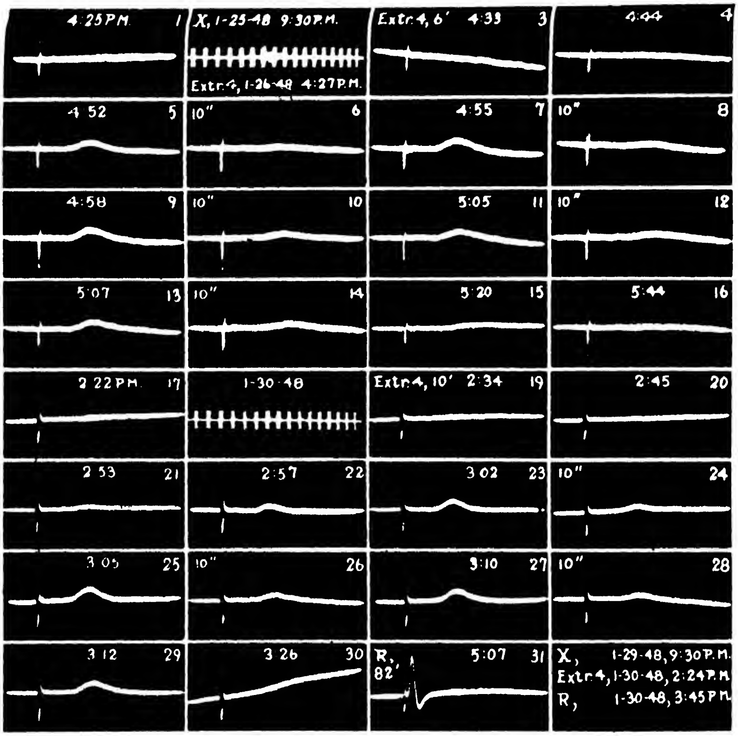


FIG. 71. Effect of brain extract 4 upon the excitability of nerve deprived of sodium. *1* and *17*, absence of conducted response in the peripheral segments of the nerves; *3* to *16* and *19* to *30*, restoration of the excitability of Et fibers by brain extract 4; *31*, spike of the fibers of slow conduction after restoration by Ringer's solution. The sweep speed and the amplification (A 50) were constant.

In both instances the conducted response increased progressively in size with advancing time (records *5* to *10* and *21* to *23*); thereafter, the response decreased progressively (records *11* to *16* and *27* to *30*). It will be noted that the speed of conduction of the restored fibers was very small; at the time when records *5* and *25* were obtained the speed of conduction was approximately 50 millimeters per second; later, it decreased progressively paralleling the increase in the spike duration. Thus, the fibers restored by brain extract 4 had properties similar

to those of fibers that have been submitted to the action of quaternary ammonium ions of the restoring type.

In the second experiment the peripheral segment of the nerve was placed in contact with Ringer's solution after it had been under the action of brain extract 4 for 81 minutes. In the presence of sodium ions the conducted response increased progressively in size and as was expected the speed of conduction underwent a spectacular increase. Record *31* presents the spike that was observed after the nerve had been in the presence of sodium ions for 82 minutes. If the brain extract had not produced irreversible changes in a number of fibers of slow conduction, a comparison of records *25* and *31* would lead to the conclusion that the brain extract had been able to restore conduction by an important number of fibers of slow conduction; there is no proof, however, that sodium ions had been able to restore all the fibers of slow conduction. No emphasis will be placed upon the number of fibers restored by the brain extract, the fact to be emphasized is that the extract did restore conduction by nerve fibers that had become inexcitable in a sodium-free medium. Emphasis may also be placed upon the similarity between the actions of brain extract 4 and of quaternary ammonium ions of the restoring type.

The experiment illustrated by figure 72 was done under slightly different conditions. After the central segment had performed its recovery in Ringer's solution a few fibers of slow conduction were found to be able to conduct impulses into the peripheral segment. This segment was kept in the presence of diethanol-dimethyl-ammonium chloride until all the fibers became inexcitable (fig. 72, *1*), and immediately thereafter the peripheral segment was placed in contact with brain extract 4. Under conditions such as these restoration by quaternary ammonium ions should be expected to produce conduction of impulses at a relatively high speed, but continued action of the restoring solution should be expected to reduce the speed of conduction (cf. section 18,b). This effect was produced by brain extract 4.

A conducted response was observed after the extract had acted upon the nerve for 17 minutes (fig. 72, *3*), and the response was observed to increase progressively in size with advancing time (records *4* to *20*). Initially (records *3*, *4*, *5*) the speed of conduction was relatively high, but it decreased progressively during the course of the experiment; at the same time the spike duration increased (records *9*, *13*, *17*). A breakdown in the amplifier prevented the observation of intermediate steps; record *21* was still distorted by amplifier noise; the noise, however, did not interfere with the observation of a lengthened spike conducted at a reduced speed. The speed of conduction still underwent a further decrease (records *22* to *26*).

The peripheral segment was finally placed in contact with Ringer's solution. Under the influence of sodium ions three changes took place (cf. records *27* to *44*), the spike height and the speed of conduction increased and the spike

duration decreased. These three are precisely the changes that sodium ions produce in nerves restored by quaternary ammonium ions. In particular, it should be noted that during the early phases of the restoration by sodium

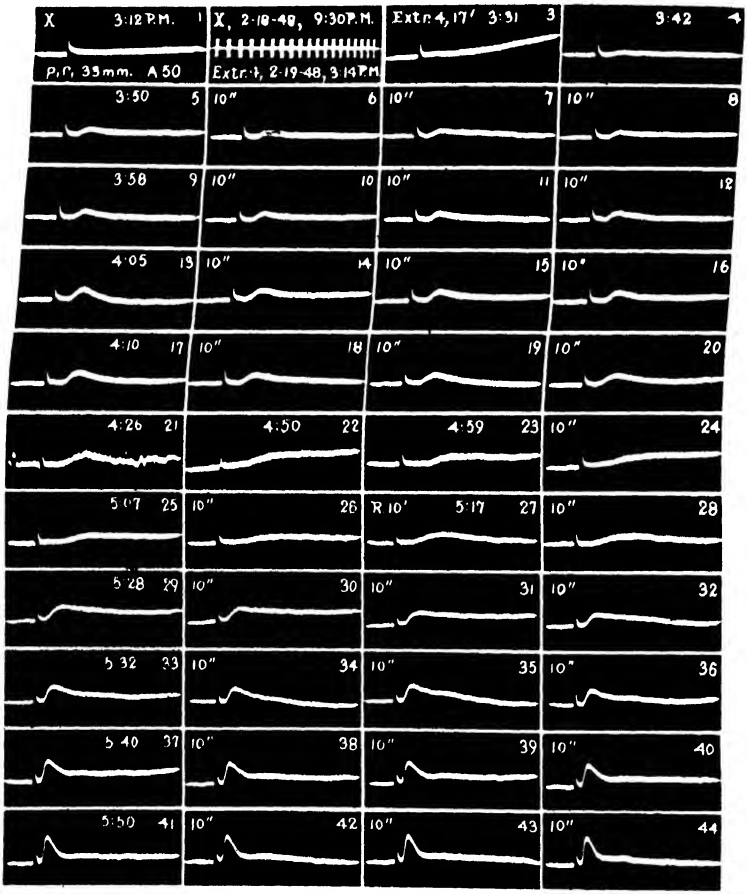


FIG. 72. Effect of brain extract 4 upon the excitability of nerve deprived of sodium. *1*, absence of conducted response in the peripheral segment of the nerve; *3* to *26*, restoration by brain extract 4; *27* to *44*, restoration by Ringer's solution.

(records *27* to *32*) the long duration of the spike indicated that the membrane potential had a very large L fraction, the deficit of which appeared as negative after-potential; during the progress of the recovery the negative after-potential decreased progressively (records *33* to *40*), until finally (records *41* to *44*) the shape of the spike approached the shape which is observed with normal nerve.

Thus, the recovery in the presence of sodium had resulted in transformation of an important part of the very large L fraction of the membrane potential into Q fraction.

Under conditions such as these it may be said that brain extract 4 had exerted upon fibers of slow conduction deprived of sodium an effect, which qualitatively was exceedingly similar to the effect of quaternary ammonium ions of the restoring type. Quantitatively, the effect produced by brain extract 4 was different from the effect produced by ethylated lysine upon the other nerve of the pair (fig. 59). The action of brain extract 4 upon the fibers of fast conduction was qualitatively different from the action of ethylated lysine, and in general, of quaternary ammonium ions of the restoring type.

The records reproduced in figure 73 illustrate the difference between the effects of ethylated lysine and of brain extract 4 upon the electrotonic potentials of frog A fibers deprived of sodium. The experiment was done with the two sciatic nerves of a bullfrog. The nerves were allowed to become inexcitable in diethanol-dimethyl-ammonium chloride. The electrotonic potentials were recorded with the arrangement of electrodes indicated in figure 3,I.

At the start of the observations the states of the two nerves were practically identical. They are characterized by records *1* to *8* of figure 73. One of the nerves (n. I) was treated with ethylated lysine (records *9* to *16*) and the other nerve (n. II) with brain extract 4 (records *17* to *24*). As was mentioned in section 17,b ethylated lysine produced only small increases in the L fraction of the membrane potential and in the polarizability of the membrane by the anodal current; nevertheless, the change in the state of the A fibers caused by ethylated lysine was in the same direction as the change that would have been produced by tetraethyl-ammonium, and in general by quaternary ammonium ions of the restoring type. Brain extract 4, however, produced a change in the opposite direction. As can readily be noted by comparing records *2* to *4* with *18* to *20* and *6* to *8* with *22* to *24*, brain extract 4 caused a decrease in the polarizability of the membrane by the anodal current.

A more drastic difference between the action of ethylated lysine and of brain extract 4 is illustrated by figure 74. Records *1* to *6* present the electrotonic potentials recorded with nerve I still in the presence of diethanol-dimethyl-ammonium ions. It will be noted that the sweep speed was considerably higher than in the case of figure 73. The details to be observed in figure 74, *1* to *6*, are these: (1) the record of the catelectrotonus (record *1*) failed to display either an E_1 deflection or the spike of an action potential at the make of the current, (2) the deflection produced by the decay of the anelectrotonus (record *2*) had a rounded corner, indicating that the break of the anodal current had failed to initiate impulses, and (3) applied polarization (records *3*, *4*) did not restore to the nerve fibers the ability to produce impulses in response to the break of

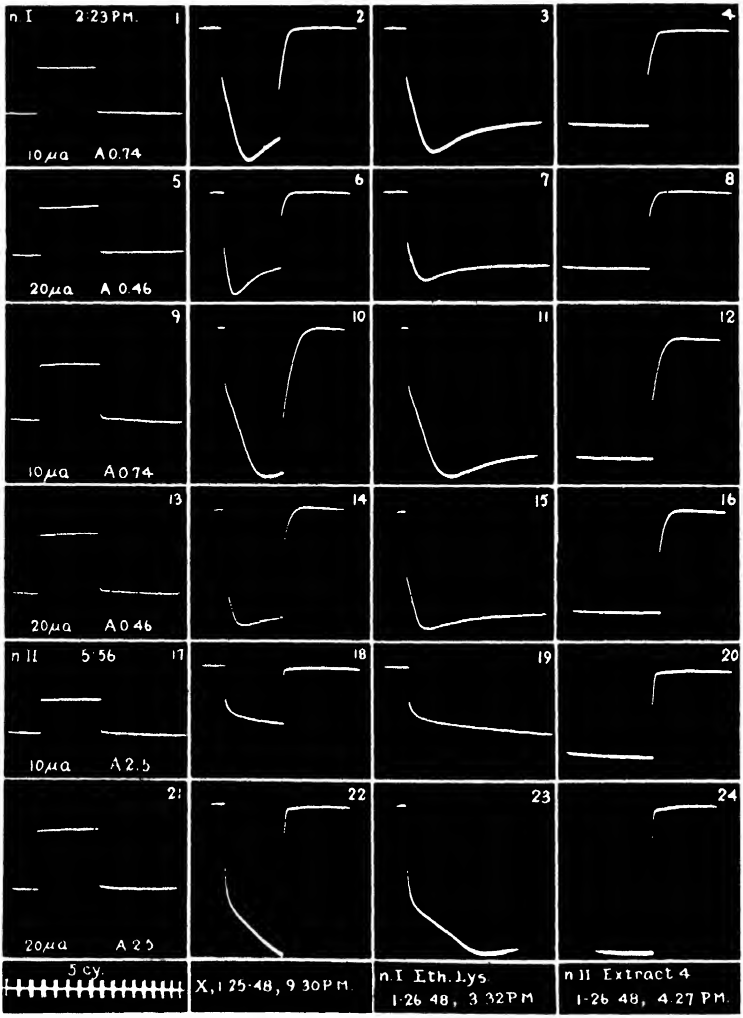


FIG. 73. Comparison of the effects of ethylated lysine and of brain extract 4 upon the electrotonic potentials produced in the peripheral segment of nerves deprived of sodium.

1 to *8*, electrotonic potentials before the application of the restoring solutions; *9* to *16*, effect of ethylated lysine; record *9* was obtained at 5:48 P.M.; *17* to *24*, effect of brain extract 4.

the anodal current (records *5*, *6*). Ethylated lysine did not improve the state of the A fibers, since the decay of the anelectrotonus has in records *7* and *8* essentially the same course that it has in record *6*.

A very different situation prevailed in the case of nerve II, that had been submitted to the action of brain extract 4. In the first place, the sharp corners of the deflections at the make of the cathodal current (record *9*) and at the make of the anodal current (record *10*) indicate that E_1 reactions of considerable intensity were produced by the A fibers. On the other hand, the applied anodal current proved to be able to improve the state of the A fibers to the extent that impulses could be initiated. The two-second period of anodal polarization used to obtain records *11* and *12* was not effective, since no impulse was initiated by the break of the short pulse of current used to obtain record *13;* but after a 6-second period of anodal polarization (records *14*, *15*) the break of the same short pulse resulted in the initiation of impulses, since in record *16* an unmistakable spike appears superposed upon the decay of the anelectrotonus. The observations were repeated with the use of a larger applied current. Whether impulses were initiated by the break of the current in the case of record *17* or not, is difficult to decide; but there can be no doubt that after applied anodal polarization (records *18*, *19; 22*, *23*) had improved the state of the A fibers impulses were initiated by the break of the current (records *20*, *21*, *24*). The shape of the recorded spikes clearly shows that the impulses were not propagated; as is observed in similar instances of restoration by anodal polarization (cf. '47, Chapter XIII), the impulses were produced only at and in the immediate neighborhood of the polarizing electrode (fig. 3,I,p_1), i.e., there, where the restoring action of the anodal current was greatest. The spike recorded at point r_1 (fig. 3,I,r_1) was referable to electrotonic spread of the spike produced at point p_1. If consideration is given to the amplification used, it is found that the spikes recorded at point r_1 were more than 1 mv high, i.e., they must have included the spikes of a significant number of A fibers.

Thus, it appears that although brain extract 4 is not able to restore A fibers deprived of sodium to the extent that conduction of impulses can occur, it induces in the A fibers a change toward normality, by virtue of which the excitable mechanism of the A fibers becomes operative after the A fibers have been submitted to the effect of an applied anodal current. In this respect the action of brain extract 4 is very different from the action of the restoring quaternary ammonium ions examined thus far.

The effect of brain extract 4 upon the excitability of A fibers has been confirmed in several other experiments. Figures 75 and 76 illustrate the results of one experiment that was done under carefully controlled conditions.

Figure 75 presents the electrotonic potentials that were recorded in the peripheral segment of the nerve before the application of brain extract 4. No sign of an E_1 deflection can be observed in cathodal record *1*, nor were impulses initiated by the break of the anodal current in the case of anodal records *2* and *4*. The two-second period of anodal polarization used to obtain records *3* and *4* resulted in a loss of polarizability of the membrane (cf. records *2* and *5*, *3* and *6*) that was augmented by the 4-second pulse used to obtain records *6*, *7* (cf. records

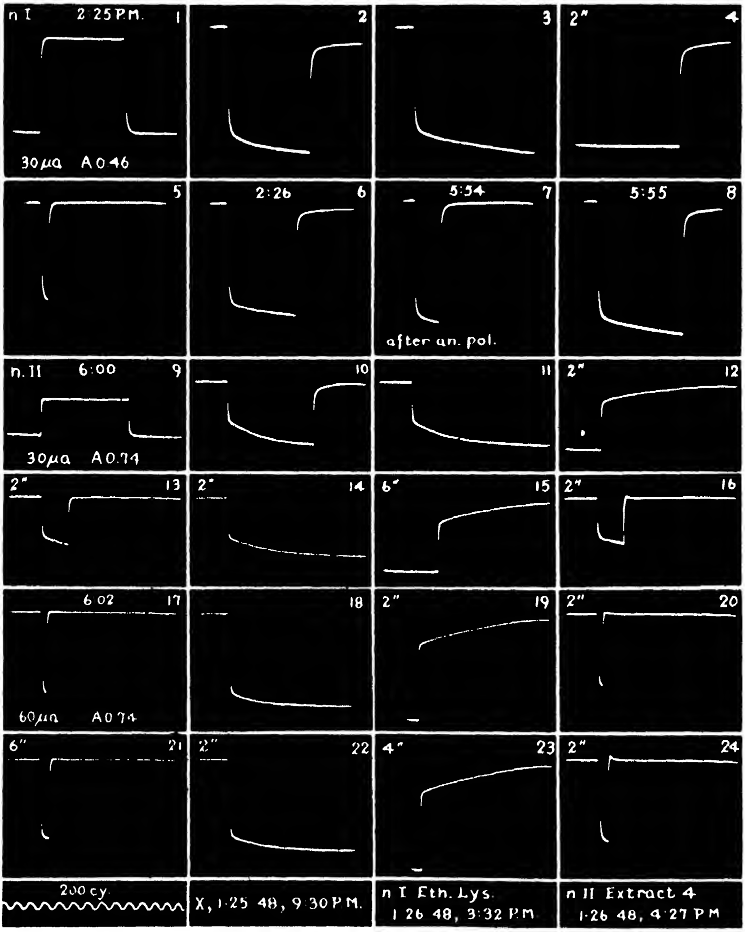


FIG. 74. Comparison of the effects of ethylated lysine and of brain extract 4 upon the electrotonic potentials produced in the peripheral segment of nerves deprived of sodium.

1 to *6*, electrotonic potentials before the application of the restoring solutions; record *5* was obtained two seconds after record *4*, and record *6*, two seconds after record *5*; *7*, *8*, effect of ethylated lysine; *9* to *24*, effect of brain extract 4. Note the spikes of A fibers superposed upon the decay of the anelectrotonus in record *16*, *20*, *21* and *24*.

5 and *8*), but the nerve fibers did not become able to produce impulses in response to the break of the anodal current. In other words, (cf. section 9,c) the decay of the anelectrotonus is faster in records *5* and *8* than in record *2* because the polarizability of the membrane had undergone a decrease as a result of the

E_3 reaction elicited by two relatively long periods of anodal polarization (records *3*, *4*; *6*, *7*), but no spikes were superposed upon the decay of the anelectrotonus

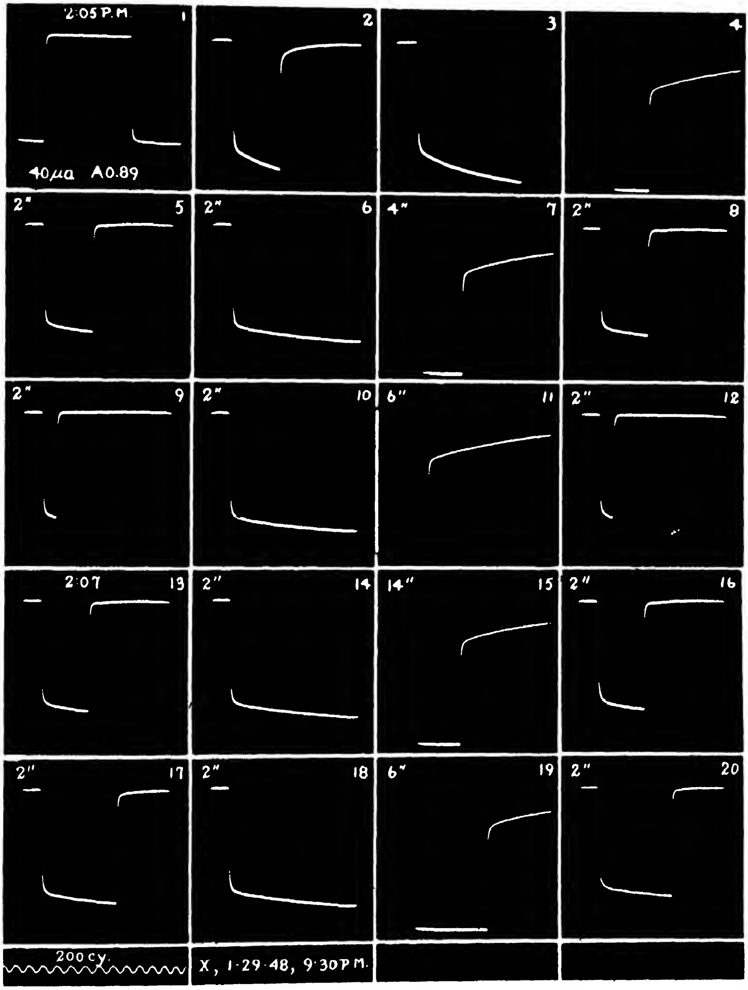


FIG. 75. Electrotonic potentials in nerve deprived of sodium. Record *4* was obtained two seconds after record *3*.

in records *5* and *8*. The situation was not altered by the use of additional periods of anodal polarization (records *10*, *11*; *14*, *15*; *18*, *19*); impulses were never initiated by the break of short pulses of anodal current (records *9*, *12*; *13*, *16*; *17*, *20*).

Brain extract 4 modified the temporal course of the catelectrotonus in an

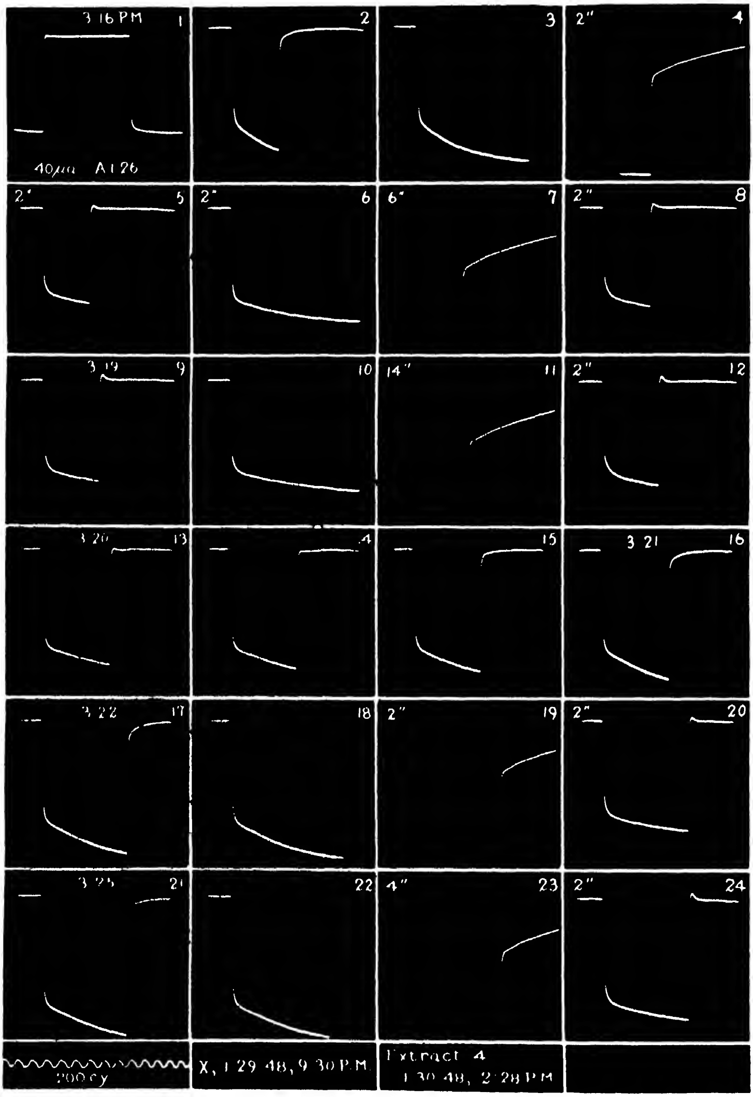


FIG. 76. Continuation of figure 75. Electrotonic potentials after restoration of the nerve by brain extract 4.

important manner. At the make of the current there appeared in the catelectrotonus (fig. 76, *1*) a sharp crest, that undoubtedly was not the spike of an action potential but an E_1 fluctuation of the electrotonic potential, since the height of the crest above the level of the catelectrotonus remained unchanged when

the magnitude of the applied current was reduced (cf. fig. 78, *16* to *21*). Therefore, brain extract 4 had restored to A fibers the ability to produce the E_1 fluctuation of the electrotonic potential, i.e., to produce a full E_1 reaction.

The break of the short pulse of anodal current used to obtain record *2* did not initiate impulses, but after the two-second pulse used to obtain records *3* and *4* the break of the short pulse initiated impulses in a number of A fibers, since a spike appears in record *5* superposed upon the decay of the anelectrotonus. A 6-second period of anodal polarization (records *6*, *7*) resulted in an increase in the break response (record *8*). The restored A fibers were maintained in excitable state by means of periods of anodal polarization, several seconds long, alternating with the delivery of short, testing pulses; records *9* to *12* illustrate the observations made at the end of this phase of the experiment. The nerve was then allowed to rest, its state being tested infrequently by the delivery of short pulses of anodal current; as is shown by records *13* to *17* the A fibers again become inexcitable, but a two-second period of anodal polarization (records *18*, *19*) restored to a number of A fibers the ability to produce impulses (record *20*). A new period of rest resulted in the disappearance of the response, but, once more, a 4-second period of anodal polarization (records *22*, *23*) restored the ability of A fibers to produce impulses in response to the break of the anodal current (record *24*).

The amount of brain extract 4 remaining after the performance of the experimental tests is so small that the isolation and the chemical analysis of the active substance would be practically impossible, for the reason that the isolation would have to be checked by experimental tests. Therefore, it was decided to prepare a large scale extract (7) using the same technique that was used in the preparation of extract 4. A small fraction of the hydrolysate was processed rapidly in order to ascertain whether or not the active substance also is present in this extract. Figures 77 and 78 illustrate the results that have been obtained.

In the experiment illustrated by figure 77, at the time when the observations were begun, a small number of fibers of slow conduction were still able to conduct impulses into the peripheral segment of the nerve (record *1*). Brain extract 7 rapidly abolished the response of those fibers (records *2*, *3*), but after the extract had acted upon the nerve for 31 minutes a larger spike was observed (records *5* to *8*) which was conducted at a markedly lower speed. The conducted response first increased in size with advancing time (records *9* to *11*, *13* to *16*) and then decreased (records *17* to *20* and *21* to *24*); the rate of decrease, however, was so small that in all probability the responding fibers would have remained excitable for a considerable length of time. Ringer's solution was observed to produce a rapid and far reaching recovery of the fibers of slow conduction (records *25* to *40*). A comparison of records *39*, *40* with records *9* and *13*

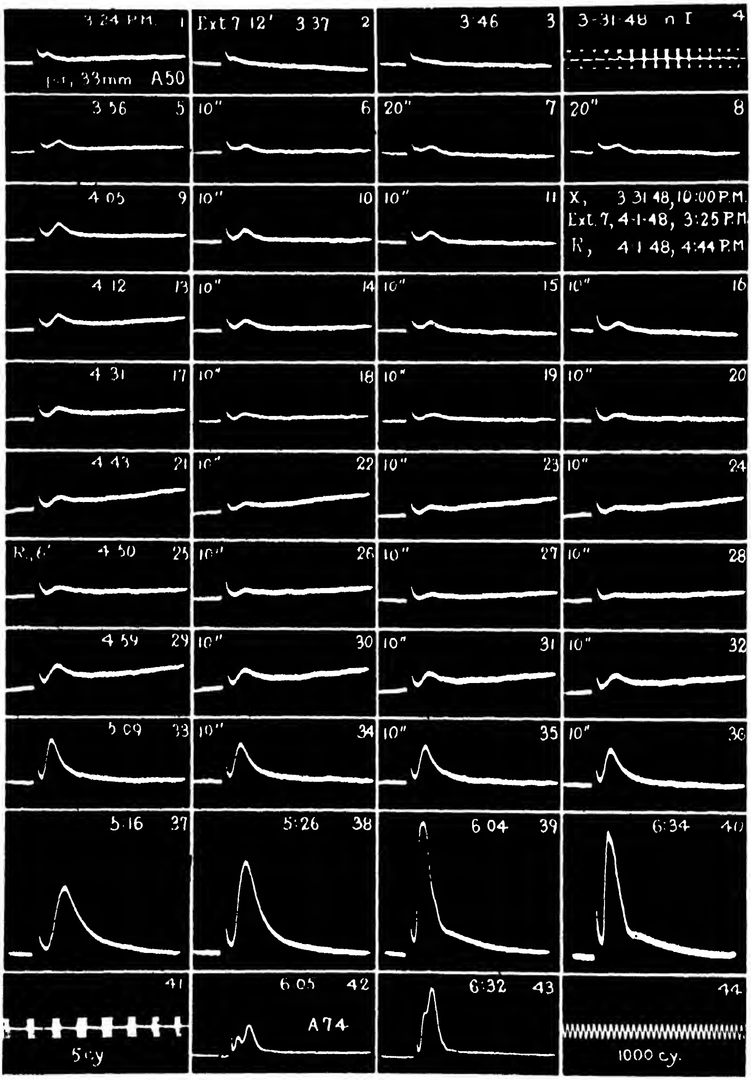


FIG. 77. Restoration of the excitability of nerve deprived of sodium. *2* to *24*, effect of brain extract 7; *25* to *43*, effect of Ringer's solution. Time line *41* applies to records *37* to *40*; time line *44* to records *42* and *43*.

shows that extract 7 had restored the excitability of only a small group of fibers of slow conduction. Ringer's solution also restored the excitability of the A fibers (records *42*, *43*).

The fact that the observations were begun at a time when a few fibers of

slow conduction still were excitable may have been one of the reasons why the restored response was conducted at a higher speed in this experiment (fig. 77) than in the experiments illustrated by figure 71. That fact, however, cannot explain why the speed of conduction remained nearly constant during continued action of extract 7 (fig. 77, *5*, *9*, *13*, *17*, *21*), while continued action of extract 4 had resulted in a marked reduction of the speed of conduction of the restored response (fig. 72, *5*, *9*, *13*, *17*, *21* to *25*). Therefore, the conclusion is unavoidable that extracts 4 and 7 have partly different actions upon frog nerve deprived of sodium.

This conclusion is also in agreement with other observations. With the nerves submitted to the action of extract 4, after sodium ions were made available to the nerves the spike of the fibers of slow conduction increased continuously in size to reach only a moderate height (fig. 71, *31*; fig. 72, *27* to *44*). The small height of the spike restored by sodium may be interpreted in two manners: (1) extract 4 had produced irreversible damage to a number of fibers of slow conduction or (2) extract 4 does not produce in the fibers of slow conduction that change which results in an increased spike height. But whichever explanation is correct, the fact is that the action of extract 4 is different from that of extract 7. After the action of extract 7 the restoration by sodium ions resulted in a prompt increase of the spike, an important feature of the restoration being this, after the spike had reached a great height (fig. 77, *39*), it began to diminish (record *40*). Therefore, although extract 7 had restored conduction by only a small number of fibers, it had caused all the fibers of slow conduction to undergo such a change, that during the restoration by sodium ions the spike temporarily acquired an increased height.

The difference between the actions of extracts 4 and 7 upon A fibers is exceedingly remarkable. Figure 78 illustrates the effect of extract 7 observed in an experiment done with the two sciatic nerves of a bullfrog. As usual the nerves were allowed to become inexcitable in 0.11 M diethanol-dimethyl-ammonium chloride.

Records *1* to *6* present the electrotonic potentials that were recorded in the peripheral segment of nerve I after all the nerve fibers had become inexcitable. There was no sign of an E_1 fluctuation at the make of the cathodal current (record *1*), nor at the make of the anodal current (record *2*). On the other hand, the break of the brief anodal pulse used to obtain record *2* did not initiate impulses; anodal polarization (records *4*, *5*) decreased the polarizability of the membrane, but it did not restore the excitability of A fibers (record *6*).

After brain extract 7 had acted upon the nerve E_1 fluctuations of the electrotonic potential were initiated by the make of the cathodal current (record *7*) as well as by the make of the anodal current (record *8*), but no impulses were initiated by the break of the anodal current, even after the nerve had been submitted to the effect of anodal polarization (record *10*). After an additional

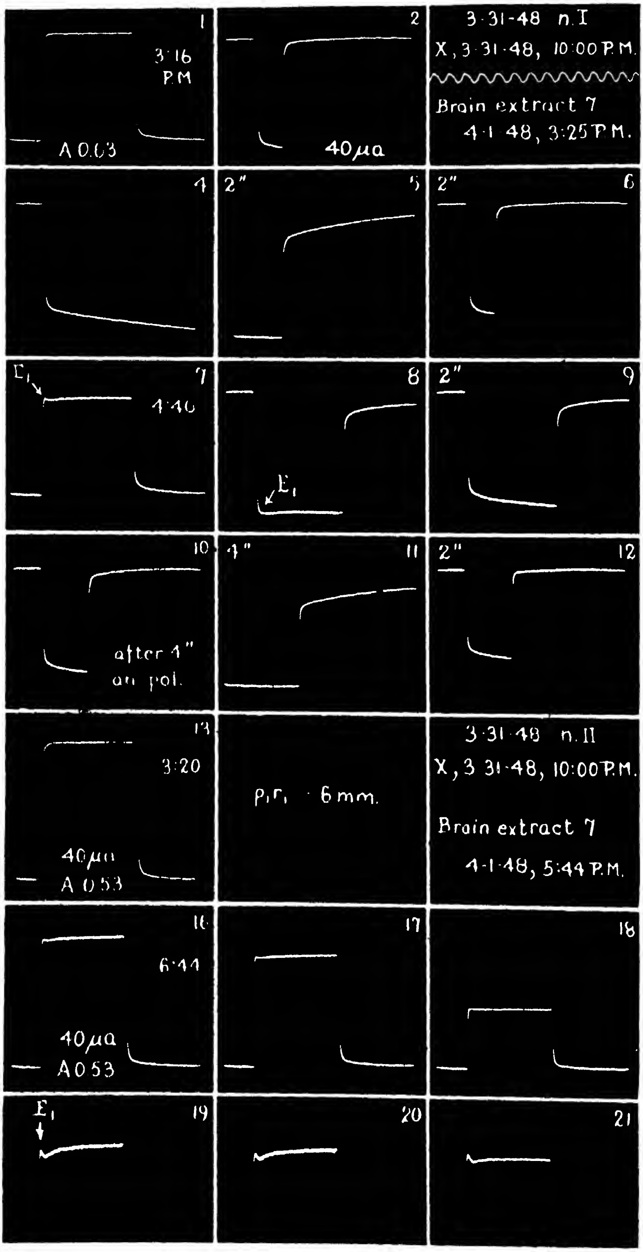


FIG. 78. Effect of brain extract 7 upon the electrotonic potential of nerve deprived of sodium.

4-second period of anodal polarization (record *11*) the decay of the anelectrotonus produced by the pulse used to obtain record *12* displayed a small sharp crest, that deserves detailed consideration.

The crest might have been produced by the spike of the action potential of a few fibers. There are, however, convincing reasons to believe that the crest was an E_1 fluctuation of the electrotonic potential, not an action potential. In the first place, the crest was not increased by the successive application to the nerve of several periods of anodal polarization; on the other hand, reduction of the applied current to 20 μa did not reduce the crest. Had the crest been an action potential, its height would have been increased by further restoration of the nerve by the anodal current, and its height would have been decreased by a reduction of the applied current.

Then, there are the following facts. A well defined E_1 fluctuation appeared in record *8* at the make of the current, while no E_1 fluctuation appeared at the break of the current. The pulse used to obtain record 8 resulted in an important change in the temporal course of the anelectrotonus; record *9* does not display an E_1 fluctuation, instead it presents the slow anelectrotonus increasing continuously during the flow of the applied current, i.e., in the manner that had been observed previously (cf. record *4*). The polarizability of the membrane was further decreased by a 4-second period of anodal polarization (record *10*), and still further by an additional 4-second period of polarization (record *12*); it was only after the polarizability of the membrane had been greatly decreased that the break of the anodal current could produce an E_1 fluctuation. On the other hand, the cathodal current was no longer able to produce an E_1 fluctuation at its make; to be more specific, after the nerve had been submitted to the effect of the anodal pulse used to obtain record *8* the catelectrotonus had a temporal course such as that which appears in record *1*. When the nerve was allowed to rest it returned to its initial state and the observations illustrated by records *7* to *12* were reproduced.

If consideration is given to the information that is available on the properties of the E_1 fluctuation ('47, Chapter VI) no difficulty is found in interpreting the situation. The E_1 fluctuation at the end of the pulse used to obtain record *8* was obliterated by the decay of the slow electrotonus; the E_1 deflection at the break of the anodal current did not become demonstrable until the polarizability of the membrane had been decreased by the E_3 reaction to such an extent that the height of the slow electrotonus became very small. After record *8* had been obtained the cathodal current could not produce E_1 fluctuations because the nerve was in the state of postanodal depression ('47, section VII.8), which is identical with the state of cathodal depression ('47, sections VI.11 and 12); the E_1 deflection could be produced only by the break of the anodal current, i.e., only after the flow of the anodal current had relieved the depression. A period

of rest also relieved the depression and for this reason, after the nerve had been allowed to rest, the observations illustrated by records *7* to *12* were reproduced.

Record *13* presents the catelectrotonus observed with nerve II before the application of brain extract 7; it is obvious that, 140 minutes before the extract was applied, the lack of sodium ions in the external medium of the nerve fibers had already resulted in the absence of the E_1 fluctuation. The catelectrotonus that was produced by the same current after restoration by brain extract 7 is presented in record *16* and at a higher amplification in record *19*. Restoration by the extract had resulted in the appearance of a full sized E_1 fluctuation. The observations were repeated with the use of smaller currents (records *17*, *20*; *18*, *21*); as can readily be observed the reduction of the magnitude of the current did not result in a reduction of the E_1 fluctuation. Indeed, the E_1 fluctuation appears with greater clarity in record *21* than in record *19* because in the case of this record the growth of the E_4 potential partly obliterated the E_1 deflection (cf. '47, sections VI.11 and 12). Since a full-sized E_1 deflection was produced by a relatively small cathodal current (record *21*), while no cathodal current, however large, was able to initiate impulses at the crest of the E_1 fluctuation, and since no impulses could be initiated by the break of the anodal current, it is clear that brain extract 7 had brought the A fibers into a very remarkable state: the fibers had been restored to the extent that they could produce E_1 fluctuations of the electrotonic potential, but they could not produce nerve impulses.[13] In this important respect the action of extract 7 is different from that of extract 4. After restoration by extract 4 the A fibers produced impulses (fig. 76); after restoration by extract 7, only E_1 fluctuations (fig. 78).

The action of extract 7 upon A fibers is illustrated again by figure 79. No E_1 deflection appears in record *1*, but a full-sized E_1 deflection is present in record *4*. No nerve impulses were initiated by the break of the anodal current either before (records *2*, *3*) or after the action of brain extract 7 (records *5* to *9*).

c. Comment. The simplest possible explanation of the differences between the actions of extracts 4 and 7 upon nerve fibers deprived of sodium is the assumption that the extracts contain two active substances (or a greater number of active substances) in different concentrations. Since the number of experiments done with the use of the extracts is sufficient to exclude the effect of differences between individual nerves (cf. section 18,b), and since the differences between extracts 4 and 7 are qualitative rather than quantitative it seems probable that the simplest explanation also is the correct explanation. For convenience, it will be assumed that extracts 4 and 7 contain only two active substances.

[13] It will be remembered that the E_1 reaction is the process that underlies the immediate restoration of losses of Q fraction of the membrane potential produced by the passage of the nerve impulse (cf. '47, section XV.4e).

The nature of the active substances is still unknown. There are several conclusive reasons to exclude the existence of a significant amount of sodium ions in the extracts: (1) the action of the extracts is essentially different from that of sodium, (2) the method of preparation includes two steps that are adequate to eliminate sodium, except in so far as sodium ions may be coprecipitated: precipitation by phosphotungstic acid and precipitation by potassium ferrocyanide, and (3) the zinc uranyl acetate reagent failed to detect the presence of a significant amount of sodium ions in extract 4. The zinc uranyl acetate test was not applied to extract 7, but the action of sodium ions upon nerve restored

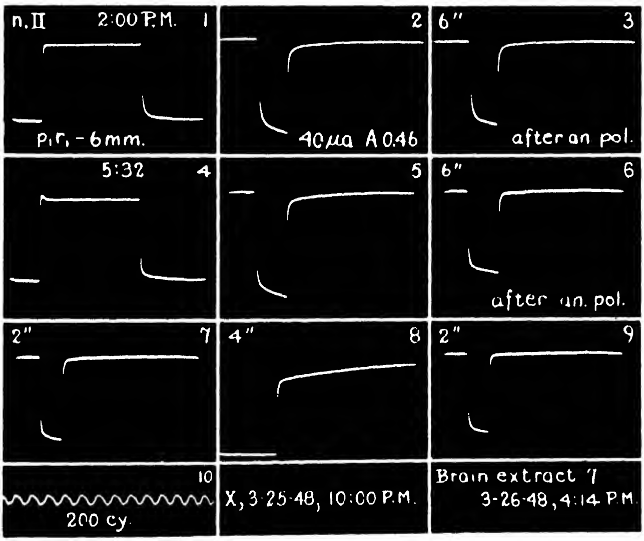


FIG. 79. Effect of brain extract 7 upon the electrotonic potential of nerve deprived of sodium.

by extract 7 (fig. 77, *25* to *40*) conclusively demonstrated that the active substance present in extract 7 was not sodium.

The substances present in the extracts at the highest concentrations are bases that are precipitated by potassium ferrocyanide from, and only from strongly acid solution. Other substances, however, must have been present in the extracts, because coprecipitation must have occurred. These impurities could have been removed by effecting a second precipitation with potassium ferrocyanide, but to do so would have resulted in the loss of a certain amount of the active substances. On the other hand, it is reasonable to believe that the activity of the extracts was due not to substances present at minute concentrations, but to substances present at a high concentration, i.e., to the bases that

were precipitated by potassium ferrocyanide. In this connection it may be stated once more that after a nerve has been deprived of sodium for an extended period of time restoration cannot be effected either by sodium ions or by quaternary ammonium ions unless the ions be present at a concentration near 0.1 N.

The nature of the active substances will not be known until a complete chemical analysis has been done. In view of the fact, however, that the precipitation by potassium ferrocyanide has a high degree of specificity for quaternary ammonium ions it seems reasonable to believe that the active substances present in brain extracts 4 and 7 are quaternary ammonium ions, of course, different from choline, acetylcholine and thiamine. Two other facts may be mentioned to support this assumption. In the first place, among naturally occurring, organic bases only quaternary ammonium ions are bases as strong as those present in extract 4. It will be remembered that extract 4 was evaporated to dryness in a vacuum at 80°C. after it had been acidified with hydrochloric acid; the residue yielded a practically neutral solution in water (pH 6.7). A similar test has not been performed with extract 7. On the other hand, the actions of extracts 4 and 7 upon nerve resemble the actions of quaternary ammonium ions of the restoring type.

To assume that the active substances of extracts 4 and 7 are quaternary ammonium ions still leaves considerable uncertainty regarding their nature, since the number of possible quaternary ammonium ions is exceedingly great. To be more specific, the actions of the extracts upon nerve lead to the conclusion that the active substances are different from the ions listed in figures 1, 2, 53 and 58, but after the exclusion of these ions the number of possible quaternary ammonium ions of the restoring type is certainly great.

Even though the nature of the active substances is unknown, the results presented in this section prove conclusively that it is possible to extract from nervous tissue (ox brains) bases that in the absence of sodium restore the excitability of frog nerve fibers that have been deprived of sodium, the restoration being, in important respects, similar to that which is effected by quaternary ammonium ions of the restoring type. In view of the experimental results it may be assumed that the bases extracted from the ox brain participate in some of the chemical reactions that underlie the regulation of the value of the membrane potential and the production of the nerve impulse. A more detailed assumption on the mode of action of the extracted bases would be hardly permissible.

20. General Considerations

a. On the working hypothesis. The experiments described in sections 16 to 19 were planned as tests of the working hypothesis presented in section 12. The hypothesis has survived the tests, but this circumstance does not prove at all that the hypothesis is correct. As the evidence stands at present the hypothesis

is permissible, but information incompatible with the hypothesis might be forthcoming. If this happens, a new hypothesis will have to be elaborated, and then probably a number of others, until finally the nature of the resting membrane potential and of the nerve impulse is understood.

On the other hand, it should be emphasized that the working hypothesis presented in section 12 is not intended to be exclusive. The hypothesis is based upon the conclusion that tetracovalent nitrogen plays an important rôle in nerve physiology; in order to give to this conclusion a vivid form, the permissible assumption was made that chemical reactions resulting in the change of tricovalent nitrogen into tetracovalent nitrogen or conversely participate in the establishment of electric double layers in the nerve membrane. Other assumptions could also be suggested. For example, the important work of Beutner and Barnes (cf. Beutner, '44; Barnes, '47) on potential differences established by quaternary ammonium ions at water-oil boundaries, might have been used to elaborate an attractive hypothesis.

b. On the mode of action of quaternary ammonium ions upon nerve. The problem presented by the differences in the actions of quaternary ammonium ions upon frog nerve is only a new aspect of an old problem, that of the relationship of chemical structure to biological action. In a masterly lecture Sir Henry Dale ('20), making specific reference to the then known differences between the pharmacological actions of tetramethyl-ammonium and tetraethyl-ammonium (cf. Burn and Dale, '15), used these words: "A few years ago, in discussing this curious contrast between the methyl and the ethyl ammonium bases, I ventured to say that it was as mysterious as the physiological contrast between sodium and potassium." No more graphic statement of the nature of the problem could be made today.

The great dependence of the properties of quaternary ammonium ions upon slight changes in the groups attached to nitrogen, and, in particular, the discontinuous changes in properties in continuous series of ions will address serious challenges to theoretical chemists who should attempt an exhaustive explanation in terms of present day information and theory. Probably the actions upon various biological materials of many series of ions with gradual structural differences will have to be investigated before the experimental information becomes sufficient for a theoretical analysis.

c. Penetration and distribution of quaternary ammonium ions. In spite of the great differences between their actions upon nerve, all the quaternary ammonium ions listed in figures 1, 2, 53 and 58 have a property in common. Their chlorides can be used, like sodium chloride, at concentrations about 0.11 M, to maintain the osmotic equilibrium of the nerve fibers. From this fact it may be concluded that the amount of quaternary ammonium ions that penetrates into the nerve fibers is only very small. The justification of the conclusion is this: If quaternary ammonium ions were able to penetrate freely into the nerve fibers,

so that their internal and external concentrations would be equal, nerves kept in 0.11 M solutions of quaternary ammonium ions would behave like nerves kept in distilled water. Maintenance of the *osmotic equilibrium is possible only if the internal concentration of the quaternary ammonium ions is small in relation to the external concentration.*

That quaternary ammonium ions can be used to maintain the osmotic equilibrium of the nerve fibers does not mean that an impenetrable barrier at the surface prevents the ions from entering into the fibers; it means only that penetration ceases when the internal concentration is a small fraction of the external concentration. Indeed, the restoring action of a number of ions upon nerve deprived of sodium and the depolarizing action of other ions are direct evidence that the large majority of the ions listed in figures 1, 2, 53 and 58 can penetrate into the nerve fibers. It is only for the few inert ions that direct proof of the ability of the ions to enter into the nerve fibers is lacking, except in one instance, that of acetylcholine (fig. 2,XXV). For the reason that the enzymatic hydrolysis of acetylcholine yields an agent, acetic acid, that exerts a deleterious action upon the nerve fibers, no difficulty is found in demonstrating that acetylcholine can penetrate into the nerve fibers, at least up to the location of the enzyme, in amounts that are sufficient to produce irreversible deterioration of the fibers ('47, section IV.8; cf. Toman, '48).

Emphasis may be placed here upon the essential difference that exists between (1) the presence of a barrier at the surface of the nerve fibers that would prevent the penetration of substances and (2) the establishment of an equilibrium between the internal and the external concentrations of a substance when the internal concentration is only a fraction of the external (cf. '47, section IV.9). The situation is best illustrated by sodium. In the case of sodium the problem consists in ascertaining why sodium, in spite of its being able to pass across the membrane and in spite of its being present outside the nerve fiber at a high concentration, is present inside the fiber only at a small concentration. This problem, the distribution problem, is one of the essential problems of nerve physiology and in general of the physiology of cells.

As the evidence stands at present every substance has to be credited with the ability to penetrate into the nerve fibers, until strict proof to the contrary has become available (cf. '47, section IV.9). That with many substances equilibrium between the internal and the external concentrations is reached when only a small amount of the substances has penetrated into the nerve fibers does not prevent the substances from exerting a profound effect upon the fibers, for the reason that an active substance need be present only at those places at which it can modify the course of metabolism and therefore of function.

21. SUMMARY

Quaternary ammonium ions that are able to restore the excitability of Et fibers deprived of sodium have been prepared by ethylation of piperidine,

1(+) lysine and histamine. The quaternary ammonium ions resulting from the ethylation of pyridine, niacinamide and coramine are not restoring ions.

Bases have been extracted from the ox brain, that probably are quaternary ammonium ions and that are able to effect a restoration of nerve fibers deprived of sodium. The base (or bases) present in brain extract 4 restores to Et fibers the ability to conduct impulses and to A fibers the ability to produce impulses after the fibers have been submitted to the effect of an applied, anodal current. The base (or bases) present in extract 7 restores to Et fibers the ability to conduct impulses and to A fibers the ability to produce E_1 fluctuations of the electrotonic potential.

The assumption is made that the bases extracted from the ox brain participate in some of those electrochemical reactions which underlie the regulation of the value of the resting membrane potential and the production of the nerve impulse.

LITERATURE CITED

ACKERMANN, D., AND F. KUTSCHER 1920 Ueber einige methylierte Aminosäuren und methylierte Aporrhegmen sowie ihr Verhalten im Tierkörper. Z. Biol., *72:* 177 186.

BARGER, G. 1914 The Simpler Natural Bases. London; Longmans, Green and Co., VIII -215.

BARNES, T. C. 1947 Phase boundary potentials of tetraethyl and tetramethyl onium compounds related to biological activity. Anat. Rec., *99:* 618.

BEUTNER, R. 1944 Bioelectricity in Medical Physics, O. Glasser, editor. Chicago, Year Book Publishers, 35-88.

BURN, J. H., AND SIR H. DALE 1915 The action of certain quaternary ammonium bases. J. Pharmacol. and Exp. Ther., *6:* 417 438.

DALE, SIR H. 1920 Chemical structure and physiological action. Johns Hopkins Hosp. Bull., *31:* 373 380.

ENGELAND, R., AND FR. KUTSCHER 1912 Die Methylierung von Histidin, Arginin, Lysin. Z. Biol., *59:* 414 419.

GUGGENHEIM, M. 1923 Biogene Amine. Handb. d. biol. Arbeitsmethoden, herausgeg. von E. Abderhalden, *1, 7:* 295 -502.

——— 1940 Die biogenen Amine. Basel and New York, S. Karger, XVI 564.

KARRER, P., G. SCHWARZENBACH, F. BENZ AND U. SOLMSSEN 1936 Ueber Reduktionsprodukte des Nicotinsäure-amid-jodmethylats. Helv. Chim. Acta, *19:* 811 828.

LORENTE DE NÓ, R. 1947 A Study of Nerve Physiology. "The Studies from The Rockefeller Institute for Medical Research." *131, 132.*

TOMAN, J. E. P. 1948 Some effects of cholinesterase upon frog sciatic nerve. Fed. Proc., *7:* 125.

VARTIAINEN, A. 1935 The action of certain new histamine derivatives. J. Pharmacol. and Exp. Ther., *54:* 265-282.

WINTERSTEIN, A. 1933 Aminosäuren. Handbuch der Pflanzenanalyse, herausgeg. von G. Klein, *4, 1:* 1-180.

[Reprinted from THE JOURNAL OF GENERAL PHYSIOLOGY, March 20, 1949, Vol. 32, No. 4, pp. 481–488]

PHAGE FORMATION IN STAPHYLOCOCCUS MUSCAE CULTURES

V. FURTHER OBSERVATIONS ON THE RELATIONSHIP BETWEEN VIRUS RELEASE AND CELLULAR LYSIS

BY WINSTON H. PRICE

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, New Jersey)

(Received for publication, October 15, 1948)

It has previously been reported from this laboratory that in synthetic medium of Fildes containing hydrolyzed casein *Staphylococcus muscae* virus is released without visible lysis of the host cell (1). Lysis of the cell did eventually occur in this system, but only after the phage was released. These observations were confirmed not only by turbidimetric readings but also by direct microscopic examination of the infected cells. It will be shown in this paper that (*a*) in a synthetic medium with a high multiple infection of virus, virus release is correlated with cellular lysis in contrast to a very low multiple infection in which virus release occurs without visible lysis although the final yield of virus is the same in both cases and (*b*) a non-dialyzable substance extracted from yeast when added to a cell infected with a very low multiple infection results in virus liberation being correlated with cellular lysis, although the final yield of virus liberated per cell is not affected.

EXPERIMENTAL RESULTS

Fig. 1 shows the effect of a low and high multiple infection on the release of the virus in synthetic medium of Fildes containing hydrolyzed casein. With a high multiple infection, virus liberation is correlated with cellular lysis. With a very low multiple infection virus liberation occurs before observable cellular lysis. The final yield of virus per cell is the same in both instances. Virus liberation begins at the same time in both instances.

The next question was whether the virus particle itself was accelerating lysis in multiple infection or whether something else in the lysate was the active lysing agent. In order to answer this question phage solutions were inactivated in three ways: (a) Shaken at 37°C. at pH 5.0, (*b*) shaken at 37°C. at pH 9.0, and (*c*) heated at 60°C. Samples were removed at short intervals. When the phage titer had dropped so that the addition of the phage solution would result in a low multiple infection, the treated solutions were added to bacterial cultures. In all three cases, the virus was liberated before cellular lysis, while in the control tube containing a sample of untreated virus which gave a multiple infection of about 7, cellular lysis occurred 30 minutes sooner and was cor-

related with virus liberation. By centrifugation at 10,000 R.P.M. for 90 minutes, it was also possible to show that the virus particle itself was the active lysing agent. Finally phage, purified by differential centrifugation as described previously (2), also caused the cells to lyse sooner when used in a high multiple infection. All the evidence therefore indicates that it is the virus particle itself which is accelerating cellular lysis under the conditions of a high multiple infection.

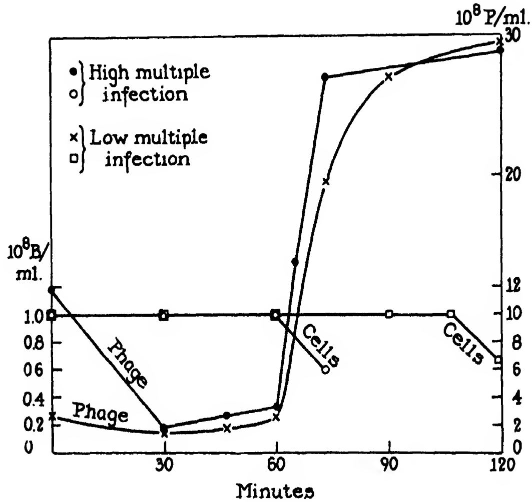


FIG 1 The effect of high and low multiple infection on the release of a bacterial virus. Two tubes, A and B, containing 10.0 ml. of synthetic medium plus 5.0 mg of hydrolyzed casein were inoculated with 1 0 $\times$ 10^8 cells prepared as described under Methods. They were incubated 1 hour at which time the cell count was 1.1 $\times$ 10^8 cells per ml. Tube A then was inoculated with virus to give 2.8 $\times$ 10^8 virus particles per ml. and tube B was inoculated with virus to give 1.2 $\times$ 10^9 phage particles per ml. Turbidimetric measurements and phage counts were then taken at intervals.

The next experiments were designed to test the theory that lysis observed in a very low multiple infection was due to the readsorption of the released particles which would then result in a high multiple infection and consequently cellular lysis. To do this experiment two tubes, A and B, were set up, with the cells being infected with a very low multiple infection. After 70 minutes, at which time all the virus had been released into the medium, the cells in tube B were centrifuged out and resuspended in fresh synthetic medium. If lysis under conditions of a very low multiple infection was due to the readsorption of released particles, then tube A should have lysed before tube B. However, both tubes lysed at the same time, cellular lysis in tube A beginning at 98 minutes and in tube B, 100 minutes. This experiment was repeated three more times with

tubes A and B lysing not more than 8 minutes apart. These experiments, together with the experiment shown in Fig. 1, in which the virus count continually rises and then reaches a plateau and remains constant, indicate that after cells have released their phage they are no longer capable of readsorbing phage. If the cells were able to readsorb phage, one would expect a drop in the virus titer sometime after the virus was released as is observed when one multiple infects normal cells (*cf.* Fig. 1, tube initially infected with a high multiple infection). This drop in titer did not occur (*cf.* Fig. 1, tube initially infected with a low multiple infection). However, more work should be done to make certain of this point.

TABLE I

The Effect of the Yeast Fraction on Virus Liberation

Two tubes, A and B, were set up containing 10.0 ml. of synthetic medium plus 5.0 mg. of hydrolyzed casein. Both tubes were inoculated with 1.0×10^8 cells per ml. Tube A received 0.2 ml. of H_2O and tube B, 0.2 ml. of yeast fraction (340 γ of N). The tubes were then incubated for 1 hour at which time the count was 1.4×10^8 cells per ml. Each tube then received 0.1 ml. of virus solution to give a virus titer of 3.8×10^8 particles per ml. Turbidimetric measurements and phage measurements were then taken every 10 minutes.

Experiment	Sample	γ N of yeast fraction per 10.0 ml.	Multiplicity of infection	Onset of lysis	Beginning of virus liberation	Virus particles liberated per cell
				min.	*min.*	
1	A	—	1.3	90–100	50–60	31
	B	340	1.6	50–60	50–60	34
2	A	—	1.6	80–90	40–50	38
	B	340	1.1	40–50	40–50	31
3	A	—	1.1	100–110	50–60	41
	B	340	1.7	50–60	50–60	36

The Effect of the Yeast Fraction

A non-dialyzable fraction was isolated from dry yeast which when added to cells infected with a very low multiple infection resulted in virus liberation being correlated with cellular lysis (Table I). It can also be seen that the yeast fraction accelerates the lysis of the infected cells. There is no effect on the final yield of virus per cell. This indicates that the yeast fraction is concerned with the lytic process and not with virus formation. The yeast fraction has no observable effect on the cells in the absence of the virus.

By varying the concentration of the yeast fraction under the condition of a low multiple infection, it is possible to vary the time of lysis as shown in Table II. It should be noted, however, that no matter at what time the cells begin

TABLE II

The Effect of Varying Concentrations of the Yeast Fraction on Cellular Lysis

The conditions were the same as described in Table I. Varying amounts of the yeast fraction were added to separate tubes. The multiplicity of infection was approximately 1.4 in all samples.

Sample	γ N yeast fraction per 10.0 ml.	Onset of lysis	Beginnnig of virus liberation	Virus particles liberated per cell
		min.	*min.*	
1	—	100–110	60–70	40
2	340	60–70	60–70	32
3	170	60–70	60–70	36
4	85	80–90	60–70	43
5	42.5	100–110	60–70	37

TABLE III

The Effect of High Multiple Infection and the Yeast Fraction on the Liberation of the Virus

Four tubes, A, B, C, and D, were set up containing 10.0 ml. of synthetic medium plus 5.0 mg. of hydrolyzed casein. All tubes were inoculated with bacteria to give 1.0×10^8 cells per ml. Tube A received 0.2 ml. of water, tube B, 0.2 ml. of yeast fraction containing 340 γ of N of the yeast fraction, tube C, 0.2 ml. of water, and tube D, 0.2 ml. of yeast fraction (340 γ of N). The four tubes were then incubated $1\frac{1}{2}$ hours. At this time the cell count was approximately 1.2×10^8 cells per ml. in all tubes. Tubes A and B were inoculated with virus to give 3.2×10^8 particles per ml. Tubes C and D were inoculated with the virus to give 1.8×10^9 particles per ml. Turbidimetric readings and phage determinations were then carried out every 10 minutes.

Experiment	Sample	γ N of yeast fraction per 10.0 ml.	Multiplicity of infection	Onset of lysis	Beginning of virus liberation	Virus particles liberated per cell
				min.	*min.*	
1	A	—	1.3	100–110	60–70	41
	B	340	1.8	60–70	60–70	36
	C	—	7.1	60–70	60–70	34
	D	340	6.3	60–70	60–70	31
2	A	—	1.7	110–120	60–70	37
	B	340	1.1	60–70	60–70	31
	C	—	6.3	60–70	60–70	44
	D	340	7.6	60–70	60–70	34
3	A	—	1.2	90–100	40–50	51
	B	340	1.8	40–50	40–50	41
	C	—	6.1	40–50	40–50	46
	D	340	7.2	40–50	40–50	48

to lyse, the yield of virus formed per cell is the same. Furthermore, in all instances, virus release begins at the same time.

Table III shows that infected cells treated with the yeast fraction behave as cells infected with a high concentration of virus. It should be noted that lysis starts approximately at the same time in the latter two instances. The addition of the yeast fraction to cells infected with a high number of virus particles does not cause lysis to occur sooner than without the yeast fraction.

In Table IV the effect of adding the yeast fraction at various times to cells with a very low multiple infection is illustrated. The sample to which the virus and yeast factor had been added at the same time began to lyse in 50 minutes. The sample to which the yeast fraction was added 45 minutes after the virus began to lyse in 90 to 100 minutes. The tube without yeast factor began to lyse in 110 to 120 minutes. This experiment shows that the yeast factor does not have to be added at the same time as the virus to influence the lytic reaction.

TABLE IV

The Effect of Adding the Yeast Fraction at Various Times on Virus Liberation from the Host Cell

Three tubes, A, B, and C, were set up containing 10.0 ml. of synthetic medium plus 5.0 mg. of hydrolyzed casein. The three tubes were then inoculated with 1.0×10^8 cells per ml. Tube A then received 0.2 ml. of water and tube B, 0.2 ml. of yeast fraction (340 γ of N). The tubes were then incubated $1\frac{1}{2}$ hours at which time the cell count was 1.2×10^8 cells per ml. All three tubes were then inoculated with virus to give 3.4×10^8 particles per ml. Phage counts and turbidimetric readings were then taken every 10 minutes. After 45 minutes, tube C received 0.2 ml. of the yeast fraction (340 γ of N).

Experiment	Sample	γ N yeast fraction per 10.0 ml.	Beginning of lysis	Beginning of virus liberation	Virus particles liberated per cell
			min.	*min.*	
1	A	—	110–120	50–60	41
	B	340 at beginning	50–60	50–60	34
	C	340 after 45 min.	90–100	50–60	30
2	A	—	100–110	40–50	51
	B	340 at beginning	40–50	40–50	49
	C	340 after 45 min.	80–90	40–50	53

Experiments were also carried out to determine whether the yeast fraction could influence cellular lysis after phage formation had stopped. Three tubes, A, B, and C, were inoculated with cells containing a very low multiple infection (about 1.3). Tube B also received the yeast fraction. At 60 to 70 minutes, when tube B began to lyse and presumably virus formation had stopped, tube C received the yeast fraction. Tubes A and C both began to lyse at 100 to 110 minutes. Experiments of this type are difficult to interpret since the time at

which the control tube A began to lyse is about when one would have expected tube C to lyse if the yeast factor could influence the lytic process after virus formation had stopped. The question whether the yeast fraction can influence cellular lysis after virus formation has stopped must remain open.

DISCUSSION

The experiments reported in this paper support the view that cellular lysis in the *S. muscae* system in synthetic medium of Fildes containing hydrolyzed casein may or may not accompany virus liberation depending upon the conditions employed in the experiment.[1] With a very low multiple infection the virus is released before the cells begin to lyse. With a high multiple infection or with the addition of a yeast fraction to cells infected with a low multiple infection, virus liberation is directly correlated with cellular lysis and occurs much sooner than the lysis observed in cells infected with a low multiple infection. The results indicate that in a high multiple infection it is the virus particle itself that accelerates the lytic process when compared to the lytic process observed in a very low multiple infection. One interpretation of this result could be that the virus contains a lytic enzyme, and thus the cells infected with a larger number of particles would lyse sooner than a cell infected with a single particle. Further speculation about the other points raised by these experiments does not appear advantageous at present.

The rôle of the yeast fraction in cellular lysis is obscure at this time. All that can be said is that it accelerates the lytic process under the condition of a very low multiple infection and does not affect the number of virus particles formed. In such a system the effect of the yeast fraction on the lytic process is similar to the effect produced by the addition of more phage particles. By adding different concentrations of the yeast fraction, it is possible to lyse the cells at varying times. No matter when the cells lyse under these conditions, the yield of virus per cell is the same within experimental error. The yeast fraction has no observable effect on the cells in the absence of the virus.

Under the above three conditions, *i.e.* cells infected with a low multiple infection, cells infected with a low multiple infection plus yeast factor, and cells infected with a high multiple infection, although the lysis time greatly varies, the final yield of virus per cell is the same within experimental error. This result is further evidence for the theory that cellular lysis is an accessory phenomenon in virus formation rather than a process directly correlated with the increase in number of virus particles formed in the cell. Evidence for such a

[1] This view also receives support by the following observation. A low multiple infection of cells in the log phase, instead of in the physiological state used in the experiments described in this paper, results in cellular lysis occurring at an earlier interval and being correlated with virus liberation.

theory has also been obtained by Fowler and Cohen (3) working with the *E. coli* system. These investigators found that the addition of certain amino acids would influence cellular lysis with little or no effect on the formation of the virus.

The fact that the virus yield per cell is the same in cells which have such varying times of lysis indicates that virus formation proceeds up to a certain point and then stops before the cells begin to lyse. This observation may mean that there is some substrate in the cell, the exhaustion of which causes virus formation to stop. The recent experiments on *E. coli* by Luria and Latarjet (4) using ultraviolet irradiation and by Latarjet (5) using x-rays could also be taken as evidence that there is some substrate in the cell, the depletion of which results in the cessation of virus reproduction.

Methods

The same *S. muscae* system was used as described previously (1). Bacteria and virus were grown and determined as described earlier (2). All calculations of virus particles formed per cell under the condition of a multiple infection are based on the phage counts after complete lysis had taken place according to the method of Delbrück and Luria (6).

The beginning of lysis as shown in Tables I to IV was determined by taking the time the first drop in turbidity was noted in the Klett-Summerson colorimeter. Since all the cells were infected in these experiments, there was no cellular multiplication and the turbidity remained constant until lysis occurred. The time of lysis determined by the turbidimetric method corresponded to the time of cellular lysis observed under the microscope.

Preparation of the Yeast Fraction.—30 gm. of Fleischmann's pure dry yeast type 20–40 was suspended in 120 ml. of distilled water and brought to 70°C. The pH was then adjusted to 5.5 with glacial acetic acid. The yeast was extracted 1 hour at 70°C. and then filtered with suction. The filtrate was dialyzed against a large volume of distilled water for 12 hours at 5°C. Further purification of the active substance could be achieved by acid precipitation at pH 4.0, with the active compound being precipitated.

This work was carried out with the technical assistance of Mr. M. Litovchick and Mr. E. Wenzlaff.

SUMMARY

1. In the synthetic medium of Fildes containing hydrolyzed casein virus release is not correlated with observable cellular lysis under conditions of a very low multiple infection.

2. In cells with a high multiple infection, lysis occurs much sooner than in cells with a low multiple infection and virus release is correlated with cellular lysis. The experiments indicate that it is the virus particle itself which accelerates lysis under these conditions.

3. A non-dialyzable fraction has been isolated from yeast, the addition of which results in cellular lysis occurring at a sooner than usual interval and being correlated with virus release in cells having a very low multiple infection.

4. By varying the concentration of the yeast fraction, it is possible to lyse the cells at varying times under conditions of a low multiple infection.

[Reprinted from THE JOURNAL OF GENERAL PHYSIOLOGY, March 20, 1949, Vol. 32, No. 4, pp. 489–502]

THE STATE OF THE CHROMOSOMES IN THE INTERPHASE NUCLEUS

BY HANS RIS AND A. E. MIRSKY

(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATES 1 AND 2

(Received for publication, November 17, 1948)

The present knowledge of chromosomes is derived mainly from a study of cells in mitosis, where they are well defined individual structures. Much less is known about the chromosomes in the non-dividing nucleus where they appear to lose their characteristic structure and individuality. In the living resting nucleus there is usually nothing visible except nucleoli. After fixation, however, a great variety of images are produced—finely or coarsely granular structures or a network of fibers with smaller or larger clumps of chromatin. Even in the same nucleus the structure can vary greatly according to pretreatment and mode of fixation. Therefore very little has been learned about interphase chromosomes from a study of fixed preparations. Since individual chromosomes cannot be recognized in the resting nucleus even the evidence that they persist from one mitosis to the next is mainly indirect. It is based on the fact that chromosomes usually do not change their position in the resting nucleus so that they reappear in prophase in the same place as they were seen at preceding telophase (5, 9).

Recently we have isolated chromosomes from resting nuclei of mammalian tissues (33). They are bodies of characteristic size and shape, visibly double, with tightly spiraled and more or less unspiraled regions. This is a direct confirmation of the view that chromosomes persist as individual structures in the interphase nucleus and this method makes it possible to study cytologically chromosomes from resting nuclei. It is at first surprising that chromosomes from interphase nuclei look so much like mitotic chromosomes, since it is usually assumed that chromosomes unravel and become completely dispersed at telophase. In mammalian tissues like thymus, liver, pancreas, kidney, this is, however, not the case. Only certain parts of the mitotic chromosomes are unraveled in the resting nucleus, others remain tightly spiralized. These thick and darkly staining regions correspond to the heterochromatic lumps seen in fixed nuclei. Structures similar to isolated chromosomes can be recognized in fixed nuclei, or nuclei isolated in citric acid, but since so many chromosomes are packed together in a nucleus they are all tangled up and cannot be seen as individual structures. The comparison of isolated chromosomes with isolated citric acid nuclei (or otherwise fixed nuclei) shows that they have not been changed essentially by the techniques of isolation, except perhaps by fragmenta-

tion. Such fragmentation cannot be common, since morphologically characteristic types of chromosomes are found repeatedly.

In the living uninjured nucleus, however, no structures can be seen as has been pointed out by many investigators of living cells. What, then, are the changes which the chromosomes undergo during isolation in physiological saline or during fixation? What is their structure in the living interphase nucleus? We find in the literature two main points of view. One places emphasis on the apparent lack of structure in the living nucleus and holds that the living nucleus does not contain formed chromosomes but two colloidal substances, chromatin and karyolymph, which are evenly dispersed. Upon fixation a separation of the two phases takes place resulting in the familiar fixation images. We may call this the *colloid hypothesis* (16, 19, 37, 40, 45). The other viewpoint emphasizes the genetic continuity of the chromosomes and holds that chromosomes exist in the living nucleus very much as they are seen in fixed preparations. They are not visible because, it is supposed, chromosomes and surrounding karyolymph have the same refractive index (21, 30, 34, 41). The best evidence for this *hypothesis of structural equivalence* of the living and the fixed nucleus comes from photographs of living cells taken with ultraviolet light of wave length 2600 Å, the absorption maximum of nucleic acid (14, 29, 43, 44). These photographs seem to show that in living cells the distribution of chromatin (*i.e.*, desoxyribonucleic acid = DNA) is the same as in fixed cells. Such photographs are, however, pertinent only if it is certain that the cells were alive and uninjured at the time when the photograph was taken. The best evidence that a cell is alive is a continued normal mitosis. Another criterion is the appearance of the nucleus in visible light, for it is well known that uninjured nuclei are usually without visible structure but that upon injury structural elements appear. Such a change in the appearance of nuclei is caused by ultraviolet radiation (4, 7). Therefore, it is necessary to demonstrate that during the exposure to ultraviolet light the appearance of the nucleus in visible light has not changed. The ultraviolet photographs of "living cells" published so far lack such a proof that the cells were not injured during preparation and exposure to the ultraviolet light. It is apparent then that the ultraviolet photographs hitherto published have given no decisive evidence on the structure of the living interphase nucleus. This has also been pointed out recently by Brumberg and Larionow (11). Using undoubtedly living cells they reached entirely different conclusions than had previous workers. Their results will be discussed later in this paper.

1. Ultraviolet Photographs of Living Cells.—In studying the chromosomes in living nuclei with ultraviolet photographs it is important to choose cells which can be demonstrated clearly to be alive and which, furthermore, have large chromosomes rich in desoxyribonucleic acid, since the absorption of chromosomes at 2600 Å depends mainly on the presence of this substance. Spermato-

cytes of grasshoppers have large chromosomes and can be followed through normal divisions in suitable preparations.

Testis follicles of a grasshopper (*Melanoplus femur-rubrum*) were teased in a drop of Belar's solution[1] (6) and the cells mounted in a hanging drop preparation. Suitable cells were then photographed at 2537 Å using a G. E. germicidal lamp (4 watt) with quartz condensing lens and a Bäckström filter[2] (1).

In such a preparation one finds among the prophase cells (pachytene) two types of different appearance. In some cells the chromosomes are highly refractile definite structures as in fixed cells, in others, on the other hand, no definite chromosomes are visible. In Fig. 2 are shown three cells form the same cyst and therefore in the same stage of prophase. In two of them the chromosomes are distinct, in the third cell only hazy shadows can be seen. A photograph at 2537 Å gives essentially the same picture, two cells with clearly visible, absorbing chromosomes, one with diffuse absorption throughout the nucleus (Fig. 1). These three cells were then irradiated with ultraviolet light (2537 Å) for 20 minutes and photographed again in visible light (Fig. 4) and at 2537 Å (Fig. 3). We see that as a result of the irradiation the nucleus which absorbed diffusely now shows distinct chromosomes both in the visible and the ultraviolet light. From a comparison of these figures it is evident that photographs at 2537 Å show definite chromosomes only when they are already distinct in visible light. Nuclei appearing homogeneous in visible light show diffuse absorption in the ultraviolet. Distinct chromosomes can be seen only in cells which are clearly injured, either mechanically during teasing or through ultraviolet irradiation. Such cells do not continue to divide. In pachytene cells which are alive and continue mitosis the nuclei appear homogeneous and absorb diffusely at 2537 Å.

Another material often used for the study of living cells is the epidermis of onion bulb scales. The epidermis can easily be stripped free and mounted on a slide in a drop of tap water. In freshly prepared epidermis only nucleoli, but no chromosomes are visible. A photograph at 2537 Å shows the diffuse absorption of the nucleus (Fig. 5). If the preparation has been standing for a while or if dilute acetic acid is added to the water, a change takes place in the nucleus. A mass of thin coiled threads becomes visible. Upon fixation with 45 per cent acetic acid the nucleus shrinks and the chromosome threads become thicker and more highly refractile. A photograph at 2537 Å gives the same picture, the absorption being localized in definite chromosome structures (Fig. 6).

Ultraviolet photography of grasshopper spermatocytes prophases and inter-

[1] NaCl (9 per cent) 20 cc.; KCl (1 per cent) 4 cc.; $CaCl_2$ (1 per cent) 4 cc.; $NaHCO_3$ (10 per cent) 0.4 cc. Distilled water up to 200 cc.

[2] The filter used with the germicidal lamp consists of a quartz cell 5 cm. in length filled with an aqueous solution of 28 per cent $NiSO_4$ and 8 per cent $CoSO_4$.

phase nuclei of onion epidermis thus gives entirely different pictures when the cells are alive than when they are injured or dead. Furthermore, ultraviolet photographs do not reveal chromosomal structures in living cells where none are seen in visible light. The difference in appearance of the nucleus when alive or dead is therefore not primarily due to a change in refractive index, but to a change in the distribution of the material with a high absorption at 2537 Å. This is the desoxyribonucleic acid (DNA) of the chromosomes. When it is distributed evenly through the nucleus as in the living cell we shall refer to such a nucleus as being in *the extended state*. When the DNA is localized in typical chromosomal structures as in fixed cells it will be referred to as *the condensed state* of the nucleus.

2. The Effect of Electrolytes and Non-Electrolytes on the Structure of the Interphase Nucleus.—That the extended state of the interphase (and also prophase) nucleus is extremely labile has been demonstrated by a number of authors since the reversible appearance and disappearance of chromatin structures was described by Lewis (27) and van Herwerden (23). Almost any interference with the cell causes the appearance of visible structures in the nucleus. In order to study the state of the chromosomes in the living nucleus and the nature of the changes upon injury, conditions must be found which will make the state of the living nucleus more stable. Cohen (17) described experiments in which nuclei of onion scale epidermis were dissected out in various media. He found that in sucrose, glycerin, and distilled water nuclei looked structureless as in the uninjured cell. Bancher (2) reported the same for glucose. On addition of salts or acid Cohen saw the chromatin structures appear as in fixed nuclei.

In order to determine whether the structure of nuclei teased in sucrose corresponds to that in living cells, grasshopper spermatocytes and onion epidermis cells were teased in 10 per cent sucrose and photographed at 2537 Å. Figs. 7 and 8 compared with Figs. 1 and 5 demonstrate the identity in appearance of living nuclei and dead nuclei in sucrose solution. If such nuclei are teased out in physiological salt solutions they have the same structure as after fixation.

If some methyl green is added to the sucrose solution the nuclei stain diffusely green. Upon the addition of acetic acid the chromosomes appear and the green stain is now limited to these structures. Since it has been shown that methyl green stains specifically the DNA of the nucleus, this is further evidence that the DNA is evenly distributed in the extended state and localized in the visible chromosomes in the condensed state of the nucleus.

Next we isolated nuclei from mammalian tissues by teasing a small piece of a drop of 10 per cent sucrose on a slide. Fig. 9 shows a rat liver nucleus photographed at 2537 Å. In sucrose the nucleus looks perfectly homogeneous. If now a drop of 0.8 per cent NaCl is added to the slide the chromatin structure appears and the nucleus shrinks (Fig. 10). If the salt is washed out with sucrose the nucleus becomes homogeneous again (Fig. 11). This process can be

repeated several times. Figs. 12 and 13 show the appearance of calf thymus nuclei in sucrose and after fixation. Figs. 14 and 15 represent beef liver nuclei in sucrose and after fixation.

The nuclei of salivary glands in Diptera are of special interest because of their peculiar banded appearance and their significance in cytogenetic work. They are rather unusual as interphase nuclei since the chromosomes are clearly visible as individual structures after fixation. If glands are dissected out in 10 per cent sucrose only the nucleolus is visible in the nucleus. By gently pressing on the cover glass the nuclei can be squeezed out of the cell. Even so they retain their homogeneous appearance. If methyl green is added to the sucrose the nuclei stain evenly green, only the nucleolar area being unstained (Fig. 23).

These observations then lead to the conclusion that the extended state of the nucleus is not peculiar to the living cell. Even in dead nuclei the chromatin can exist either in an extended state or condensed into chromosomal structures depending on the medium. In non-electrolytes (glucose, sucrose, glycerin) the chromatin is extended, in electrolyte solution it condenses.

3. The Effect of Electrolytes and Non-Electrolytes on Isolated Chromosomes.— What is the nature of this reversible change in the distribution of DNA in the nucleus? Does the DNA go on and off the chromosomes or do the chromosomes themselves swell and contract?

To answer this question chromosomes from calf thymus resting nuclei were isolated in 0.8 per cent NaCl and suspended in sucrose. Even in the test tube the difference in appearance of the chromosomes in salt and sucrose is striking. The suspension of chromosomes in sucrose is much less opaque than in saline and greater centrifugal force is necessary to spin them down. Under the microscope individual chromosomes are almost invisible. If some methyl green is added they stain green and one can see now that they have the same general shapes but are greatly swollen (Fig. 17). Upon addition of 0.8 per cent NaCl they shrink and look again as they did originally (Fig. 16). As with nuclei the change is reversible. This change in volume of the chromosomes can be measured directly. Equal amounts of isolated chromosomes were suspended in 0.8 per cent NaCl and in 30 per cent sucrose. They were then centrifuged down until no further change in volume of the chromosome mass took place. In sucrose the volume of the precipitate is four to five times greater than in saline. The swelling and shrinking which were observed on nuclei in sucrose and salt solutions (Figs. 9–11) is therefore the result of the volume changes in the chromosomes. When the chromosomes condense the nucleus shrinks and the nuclear membrane often looks shrivelled (Fig. 10). If the chromosomes are made to extend again they fill the nucleus and expand it so that the nuclear membrane becomes tight and smooth. Analysis of chromosomes in saline and after standing in sucrose overnight shows that no DNA comes off the chromosomes when

they swell (Table I). Some pentose phosphorus is lost, caused by slight autolysis of the residual chromosome (33).

The behavior of the chromosomes in sucrose was then compared with that of residual chromosomes (*cf.* reference 33), that is to say chromosomes from which the histone and practically all the DNA have been removed. These residual chromosomes look the same whether they are suspended in sucrose or saline. This is further evidence that the DNA is responsible for the change in the state of the chromosomes.

These experiments on isolated chromosomes demonstrate clearly that the change observed in the appearance of nuclei is actually a change in the state of the individual chromosomes themselves. The highly polymerized DNA which makes up a large part of most chromosomes forms a gel-like structure which can reversibly extend and condense.

4. The Effect of Salt Concentration of Interphase Chromosomes.—If interphase chromosomes isolated from mammalian tissues are suspended in 1 M NaCl a

TABLE I

Total Nucleic Acid P and Pentose P of Two Preparations of Calf Thymus Chromosomes in Saline and after Being Suspended in 30 Per Cent Sucrose Overnight

Total nucleic acid P in per cent dry weight		Pentose P in per cent total nucleic acid P	
Saline	Sucrose	Saline	Sucrose
3.75	4.04	2.2	0.57
4.05	4.21		

thick gel is formed. Microscopic study reveals that this gel is due to the swelling of the individual chromosomes. Upon rapid stirring the gel breaks and the DNA and histone go into solution. Nuclei of onion scale epidermis behave similarly. If teased out in 1 M NaCl the nuclei become homogeneous, they swell until the membrane breaks, and the nucleolus floats out into the cytoplasm. Sometimes the nucleus swells considerably before bursting. The nuclear contents then separate into two fractions, a cap-like mass of threads and a clear area (*Kappenplasmolyse*, *cf.* references 3, 40). The fibrous mass consists most likely of the residual chromosomes, while in the clear vacuole the DNA and histone are in solution.

If nuclei are fixed in acetone followed by 95 per cent alcohol the DNA condenses, but in 1 M NaCl the nucleus becomes again homogeneous, staining diffusely with methyl green. After several hours in 1 M NaCl in the cold the nuclei no longer stain with methyl green, which indicates that the DNA has gone into solution.

The effect of salts on nuclei and chromosomes then depends on the concentration of the salts. In very low concentrations the chromosomes are in the ex-

tended state, in physiological concentration they condense. In higher concentration they extend again until finally the DNA goes into solution. The behavior of nuclei and chromosomes thus parallels the behavior of isolated nucleohistone (32).

5. *Fixation of Nuclei in the Extended State.*—After treatment with most common fixatives the interphase nuclei show definite structure; the chromatin is fixed in the condensed state. But it has been observed many times that the structure of interphase nuclei varies with different fixatives. After fixation with formalin and osmic acid for instance the nuclei were often found to be quite homogeneous. Formalin and osmic acid thus preserve the extended state of the uninjured living nucleus. To prevent occasional condensation after formalin fixation we found the following method most useful. Before fixation it must be made certain that the nuclei are in the extended state, either in uninjured cells or, with dead material, in sucrose. The material is then fixed in 20 per cent formalin (1 part neutral formalin plus 4 parts water). Time of fixation depends on the size of the material. The formalin is replaced with 0.2 M lanthanum acetate for several hours. After careful washing with water the nuclei can be stained with Feulgen.

Fig. 18 shows a nucleus of onion scale epidermis fixed with formalin and stained with Feulgen. The nucleus stains diffusely and only the nucleoli are unstained. The cytoplasm and the nucleoli are perfectly clear. Fig. 19 represents a nucleus which was in the condensed state before fixation, treated in the same fashion. The chromosome threads are clearly visible. In Fig. 21 we see a pachytene of the grasshopper fixed in the extended state and stained with Feulgen. The Feulgen picture is identical with the ultraviolet photograph of a living nucleus (Fig. 1). The substance absorbing diffusely at 2537 Å in a living nucleus is therefore DNA. Isolated chromosomes in sucrose can be fixed in the extended state by adding formalin to the sucrose suspension.

6. *Distribution of Protein in the Extended State.*—After the nuclei are fixed in the extended state a cytochemical test for proteins can be performed (35). Only the distribution of total protein can be shown, since after fixatives which preserve the extended state the histone can no longer be removed with acids.

Fig. 20 shows a nucleus of the onion scale epidermis, fixed in the extended state, treated with trichloracetic acid-Millon reagent and photographed at 3650 Å. A marked general absorption of the nucleus and the absence of structure are apparent. Therefore, in the extended state we find not only the DNA but also protein, diffusely distributed through the nucleus.

7. *The Behavior of Chromosomes with Low DNA Concentration.*—If the reversible extension and condensation of chromosomes are due to their DNA content, then we should expect that chromosomes with a very low concentration of DNA would behave differently from the chromosomes discussed so far. The lampbrush chromosomes in the oocyte of the frog contain so little DNA that

they hardly stain with Feulgen or methyl green. Germinal vesicles of frog eggs were dissected out in 10 per cent sucrose. The chromosomes are faintly visible as delicate threads forming the characteristic loops. If the nuclei are dissected out in sucrose to which a little pyronin is added, the chromosomes begin to stain red and stand out most clearly (Fig. 24). Since pyronin in high concentration precipitates nucleic acid, the solution used was tested on a piece of calf liver. The liver nuclei, teased in this solution, remained completely extended. The pyronin, therefore, did not cause the appearance of chromosome threads. Germinal vesicles dissected out in sucrose were also fixed with 20 per cent formalin. *The lampbrush chromosomes were as clearly and distinctly visible as after fixation in Carnoy (alcohol-acetic acid). Furthermore, the fixation did not distort the appearance of the chromosomes, they looked no different from those in sucrose.*

Chromosomes poor in DNA thus do not show reversible condensation and their structure is not visibly altered through handling or fixation. They behave in the same way as residual chromosomes prepared from isolated calf thymus chromosomes.

We can now summarize the evidence that the DNA is responsible for the reversible extension and condensation of chromosomes. In living nuclei we have found a diffuse absorption throughout the nucleus at 2537 Å. Nuclei isolated in sucrose stain evenly with methyl green. After fixation with formalin or osmic acid the nuclei stain diffusely with Feulgen. Thus in the extended state the DNA is distributed throughout the nucleus. Finally residual chromosomes and lampbrush chromosomes which contain very little DNA do not show the reversible condensation.

8. The Nuclei of Chick Fibroblasts in Tissue Culture.—In 1946 Brumberg and Larionow (11) published some very interesting ultraviolet photographs of living and dead cells in tissue culture. Using a reflecting objective they did not have to expose the cells to the ultraviolet except in taking the photograph. They found that the nuclei of living cells absorb very little at 2600 Å. Only after the cells were killed by longer exposure to the ultraviolet did nuclear structures become visible which appeared to absorb at 2600 Å. They concluded that the DNA either did not absorb in the living nucleus or that it was differently distributed.

We have repeated and extended these observations on cultures of chick embryo fibroblasts.[3] In order not to expose the cells unnecessarily to the ultraviolet the focussing was done in visible light and then adjusted for 2537 Å by moving the fine adjustment a definite number of units which had been determined empirically. We found that after taking a photograph at 2537 Å the cells went through normal mitosis and were therefore alive at the time of exposure. A group of cells were then killed by prolonged exposure to the ultraviolet and photographed again. The pictures of living cells were found to be

[3] These cultures were kindly prepared for us on quartz slides by Dr. Ruth Hoffman.

strikingly different from those of cells killed with ultraviolet. In living fibroblasts the cytoplasm appears dark, the nucleus much lighter and without structure except for nucleoli. In the dead cells, however, the cytoplasm is very light, but the nucleus now stands out clearly with definite membrane and chromosomal structure. So far then our observations agree with those of the Russian workers. But it must now be determined whether the darker appearance of the dead nucleus is due to specific absorption at 2537 Å as Brumberg and Larionow assumed or caused by an increase in structural light loss due to a change in refractive indexes. Nucleic acids can be removed in cytological preparations by heating at 90°C. in 0.3 M trichloroacetic acid for 15 minutes (35). A culture of chick fibroblasts was therefore fixed in acetic-alcohol and a group of cells photographed at 2537 Å. The nucleic acids were then removed in hot trichloroacetic acid and the same cells photographed again at 2537 Å. After the trichloroacetic acid treatment the nucleoli and the cytoplasm were markedly less dark, but the appearance of the chromatin had not changed. It follows that some of the dark appearance of the cytoplasm and nucleoli was due to nucleic acid. The appearance of chromatin structures in the dead nucleus, however, is not caused by specific absorption, but by structural light loss due to a change in refractive indexes. This means that the nucleus of these cells contains little DNA, while the cytoplasm is rich in ribosenucleic acid. Therefore, the nucleus is lighter than the cytoplasm in ultraviolet photographs of living cells. When a cell is killed with ultraviolet the ribosenucleic acid leaks out of the disintegrating cytoplasm and the refractive index of nuclear membrane and chromatin increases over that of the surrounding medium. Therefore, in ultraviolet photographs the nucleus appears now darker than the cytoplasm and with definite internal structure. Brumberg and Larionow were certainly correct in their observations that ultraviolet photographs of living and dead cells are strikingly different. But the cells they chose contain so little DNA that they are unsuited for the study of the nature of any changes in the distribution of nucleic acids. Nuclei with a higher concentration of DNA had to be used for that.

9. The State of Chromosomes during Mitosis.—During nuclear division, when the chromosomes are moved about in the cell, they occupy only a small volume of the nucleus. Since they fill the entire nucleus during interphase, they must decrease in volume in addition to the spiralization which occurs during prophase. Such a decrease in volume can be effected easily through a condensation of the DNA during mitosis. In living cells the chromosomes are usually visible during metaphase and anaphase. The change in appearance during fixation is small compared with that of the resting nucleus. This indicates that indeed a condensation of the DNA has taken place. But it is only partial, since a further condensation can still occur in mitotic chromosomes with weak acids, or through asphyxiation, without killing the cell. In fresh hanging drop preparations of grasshopper spermatocytes the chromosomes are faintly visible.

After some time the chromosomes become more refractile and sharply outlined. The same effect is observed if the pH of the medium is lowered with CO_2 or dilute acetic acid (pH 5–6). Such cells can still finish mitosis normally.

Fig. 22 shows a metaphase of the first spermatocyte division fixed with formalin-lanthanum acetate and stained with Feulgen. It comes from the same preparation as Fig. 21 which shows a pachytene nucleus in the extended condition. The metaphase chromosomes appear somewhat swollen and with hazy outlines compared with similar chromosomes fixed in acid fixatives.

In prophase chromosomes therefore a partial condensation of the DNA takes place together with the coiling of the chromonemata. During telophase this is reversed again into the maximally extended state of the interphase nucleus.

DISCUSSION

The discoverer of the nucleus, Robert Brown, described it as a clear vesicle within every cell. Since then many investigators studying living cells in both animals and plants found the nucleus to be without visible structure except for the nucleoli. The use of fixatives and dyes allowed a detailed analysis of the morphology and the complicated behavoir of the chromosomes during cell division. But only conflicting results were obtained with regard to the structure of the resting nucleus, especially since little was known about the changes which take place in it during fixation. Engelmann (20) and Flemming (21) already knew that even unfixed nuclei could look quite differently depending on the medium. The reversible appearance and disappearance of chromosome structures in the living nucleus were first clearly described by Lewis (27) and van Herwerden (23) and since then a large number of papers have been published describing the various conditions under which structure appears in previously homogeneous nuclei. It was thus established that structures seen in fixed preparations are invisible in uninjured nuclei. This situation bas been explained mainly in two ways: (1) The *colloid hypothesis* assumes that the chromatin exists in the resting nucleus in colloidal dispersion and not in individually persisting chromosomes (16, 19, 36, 40, 45). The evidence for this view is as follows: Agents causing the appearance of nuclear structures are also coagulants of nucleoproteins. The nucleus can be fixed in the extended state with osmic acid or formalin and stained with Feulgen. Such preparations show the DNA in these nuclei to be evenly distributed throughout the nucleus (17, 31, 42). In concentrated salt solutions the nuclear content is separated into two phases, karyolymph (sol) and chromatin (gel) (microdissection experiments of Strugger (40) and Bancher (3)). Microdissection shows that most nuclei are filled with a highly viscous substance, but not with chromosomal bodies (4, 26). (2) The *equivalence hypothesis* on the other hand holds that chromosomes exist in the living nucleus as individual structures in a more or less despiraled state and similar to the way they are seen in fixed and stained cells. It is based mainly on the evidence of genetic continuity of chromosomes and observations

on the position of chromosomes in telophase and following prophase (5, 9). Ultraviolet photographs of assumedly living cells showing typical chromatin structures absorbing at 2600 Å were thought to be definite proof for this point of view.

There can hardly be any serious doubt today that chromosomes are persistent structures and that changes in their individuality are rare events caused by spontaneous breakage or under experimental conditions (x-ray, etc.). But the cytological evidence has been indirect only, since it is usually impossible to recognize individual chromosomes in the non-dividing nucleus. With the preparation of morphologically distinct chromosomes from mammalian tissue cells the direct cytological evidence for the individual persistence of chromosomes has been produced. The problem now was no longer whether chromosomes persist during interphase, but in what state they exist in the living nucleus and what changes they undergo upon fixation. Ultraviolet photographs of uninjured cells and the Feulgen staining of formalin-fixed nuclei show that the colloid hypothesis was correct in assuming an even distribution of DNA in the nucleus. But the behavior of isolated chromosomes in electrolyte and non-electrolyte solutions demonstrates that this does not contradict the assumption of individual persistence of the chromosomes. Depending on the state of the highly polymerized DNA each chromosome exists either in an extended or condensed form. Nucleohistone itself behaves in a similar fashion towards electrolytes and non-electrolytes as was pointed out by Jeener (24) who therefore suggested that the properties of nucleohistone might explain the various aspects of nuclei and chromosomes. But the nucleus and the chromosomes are not simply gels of nucleohistone. Chromosomes consist of a complex system of non-histone protein, DNA, and histone with a definite structure. Properties of chromosomes can therefore be studied only on intact chromosomes and not on nucleohistone gels or nucleohistone fibers. Thus, even though the behavior of chromosomes in the living cell can be imitated with nucleohistone in various concentrations of inorganic ions, we know nothing as yet about the conditions in the living nucleus which cause the chromosomes to extend or condense, nor about the possible meaning of the extended state for the functioning of the chromosomes in the metabolic nucleus.

During mitosis, when the chromosomes become tightly coiled, a partial condensation of the DNA takes place. Therefore, chromosomes are generally visible in living dividing cells. Agents which cause condensation in interphase nuclei can cause a further condensation and therefore an increase in refractility of mitotic chromosomes. This explains the observations of cytologists who found that the visibility of chromosomes changes with pH, tonicity of the medium, mechanical injury, etc. (8, 12, 36). In telophase, together with the despiralization, the chromosomes extend again, so that they all touch each other and can no longer be seen individually. This process has been observed many times in living cells (*cf.* reference 28) and the chromosomes were said to swell

into separate vesicles. In some cases, especially during cleavage, such separate chromosome vesicles or karyomeres are clearly visible during interphase and some authors concluded from this that in every resting nucleus the chromosomes exist as vesicles, even if no membranes can be seen (25, 28). Karyomeres in prophase, however, show clearly that the karyomere membrane behaves like a nuclear membrane and not like part of the chromosome. The chromosome condenses and spiralizes inside the karyomere membrane which breaks down in later stages of mitosis (*cf.* reference 18). Whether a membrane is formed around a chromosome group or around individual chromosomes probably depends on whether the chromosomes are close together or widely separate at telophase. In any case the chromosomes of the resting nucleus are not vesicles surrounded by a membrane.

The state of the chromosomes in the interphase nucleus naturally determines its viscosity. In most tissue nuclei with a high concentration of DNA the chromosomes fill the entire nucleus. Therefore, nuclei were found to be filled with a highly viscous mass which could be pulled into fibers with the micromanipulator (4, 26). Egg nuclei, however, were described as containing a liquid of low viscosity (22). These are nuclei with a very low concentration of DNA and where the chromosomes fill only a small part of the nuclear volume.

Stedman and Stedman (38) have recently broached the hypothesis that DNA exists mainly in the nuclear sap and does not form an integral part of the chromosome. Their view was based on an unusual interpretation of the Feulgen reaction. With regard to this they have been answered adequately (10, 13, 15, 39). But it must be pointed out here that the even distribution of the DNA in the living nucleus demonstrated in this paper is entirely different from Stedman's assumption. We have shown that the DNA forms an integral part of the chromosomes and cannot be dislodged from them without breaking chemical bonds, and that the uniform distribution is the result of the swelling of the individual chromosomes, so that they fill the entire volume of the nucleus.

SUMMARY

In the living interphase nucleus no chromosomal structures are visible. Yet in the injured cell and after treatment with most histological fixatives chromatin structures become apparent. Under certain conditions this appearance of structure in the living interphase nucleus is reversible.

We have found that this change in the interphase nucleus is the result of a change in the state of the chromosomes. In the living nucleus the chromosomes are in a greatly extended state, filling the entire nucleus. Upon injury the chromosomes condense and therefore become visible. At the same time the nuclear volume decreases. This behavior of the chromosomes is connected with their content of desoxyribonucleic acid (DNA). This view is based on the following observations:

(*a*) *Distribution of DNA in the Nucleus.*—(1) The living interphase nucleus

of uninjured cells absorbs diffusely at 2537 Å. No chromosomal structures are visible in ultraviolet photographs unless they are also distinct in ordinary light. If the chromosomes are made to condense they become visible and the absorption at 2537 Å is now localized in these structures. (2) After fixation with formalin and osmic acid interphase nuclei stain diffusely with Feulgen. These fixatives preserve the extended state of the chromosomes. (3) If nuclei are teased out in non-electrolytes (sucrose, glycerin) the chromosomes are extended. Such nuclei stain homogeneously with methyl green. On adding salts the chromosomes condense and the methyl green is now restricted to the visible structures.

(*b*) *Extension and Condensation of Isolated Chromosomes.*—When chromosomes isolated from interphase nuclei of calf thymus are suspended in sucrose, their volume is four to five times larger than in saline, but they retain their characteristic shapes. Chromosomes from which DNA and histone have been removed do not show this reversible extension and condensation, neither do lampbrush chromosomes of frog oocytes which contain very little DNA.

During mitosis a partial condensation of the DNA occurs in prophase, so that the mitotic chromosomes now occupy a much smaller volume of the nucleus. At telophase the chromosomes swell again to fill the entire nucleus.

BIBLIOGRAPHY

1. Bäckström, H. L., *Ark. Kemi, Min. Geol.*, 1940, **13A,** 1.
2. Bancher, E., *Protoplasma*, 1938, **31,** 301.
3. Bancher, E., *Biol. Gen.*, 1939, **14,** 293.
4. Bancher, E., *Protoplasma*, 1942, **36,** 607.
5. Belar, K., *Arch. Protistenk.*, 1926, **53,** 312.
6. Belar, K., *Arch. Entwcklngsmechn. Organ.*, 1929, **118,** 359.
7. Biebl, R., *Protoplasma*, 1942, **36,** 491.
8. Boche, R., and Buck, J. B., *Physiol. Zool.*, 1942, **15,** 293.
9. Boveri, T., *Arch. Zellforsch.*, 1909, **3,** 181.
10. Brachet, J., *Experientia*, 1946, **2,** 142.
11. Brumberg, E. M., and Larionow, L. T., *Nature*, 1946, **158,** 663.
12. Buck, J. B., and Boche, R. D., *Biol. Bull.*, 1938, **75,** 344.
13. Callan, H. G., *Nature*, 1943, **152,** 503.
14. Caspersson, T., *Skand. Arch. Physiol.*, 1936, **73,** suppl. 8.
15. Caspersson, T., *Nature*, 1944, **153,** 499.
16. Chambers, R., in General Cytology (E. V. Cowdry, editor), Chicago, The University of Chicago Press, 1924, 265.
17. Cohen, I., *Protoplasma*, 1937, **27,** 484.
18. Cooper, K. W., *Chromosoma*, 1939, **1,** 51.
19. Della Valle, P., *Z. Chem. u. Ind. Kolloide*, 1913, **12,** 12.
20. Engelmann, T., *Jenaische Z. Naturwissensch.*, 1868, **4,** 321.
21. Flemming, W., Zellsubstanz, Kern- und Zellteilung, Leipzig, Vogel, 1882.
22. Harris, J. E., *Brit. J. Exp. Biol.*, 1939, **16,** 258.
23. van Herwerden, M. A., *Biol. Zentr.*, 1924, **44,** 579.

24. Jeener, R., *Compt. rend. Soc. biol.*, 1946, **140**, 689.
25. Kater, J. McA., *Quart. J. Micr. Sc.*, 1929, **72**, 189.
26. Kite, G. L., *Am. J. Physiol.*, 1913, **32**, 146.
27. Lewis, M. R., *Bull Johns Hopkins Hosp.*, 1923, **34**, 373.
28. Lewis, W. H., *Anat. Rec.*, 1948, **97**, 433.
29. Lucas, F. F., and Stark, M., *J. Morphol.*, 1931, **52**, 91.
30. Martens, P., *Compt. rend. Acad. sc.*, 1927, **184**, 615.
31. Makarov, P. V., *Compt. rend. Acad. Sc. U. S. S. R.*, 1946, **54**, 69.
32. Mirsky, A. E., and Pollister, A. W., *J. Gen. Physiol.*, 1946, **30**, 117.
33. Mirsky, A. E., and Ris, H. *J. Gen. Physiol.*, 1947, **31**, 1.
34. Nemec, J. B., *Protoplasma*, 1929, **7**, 423.
35. Pollister, A. W., and Ris, H., Cold Spring Harbor Symposia on Quantitative Biology, Cold Spring Harbor, Long Island Biological Association, 1947, **12**, 147.
36. Sakamura, T., *Protoplasma*, 1927, **1**, 537.
37. Schaede, R., *Protoplasma*, 1928, **3**, 145.
38. Stedman, E., and Stedman, E., *Nature*, 1943, **152**, 267.
39. Stowell, R. E., *Stain Technol.*, 1946, **21**, 137.
40. Strugger, S., *Protoplasma*, 1930, **10**, 363.
41. Tischler, G., Allgemeine Pflanzenkaryologie, Berlin, Borntraeger, 1922.
42. Voss, H., *Z. mikr.-anat. Forsch.*, 1933, **33**, 222.
43. Wyckoff, R. W., and Ter Louw, A. L., *Science*, 1931, **74**, 664.
44. Wyckoff, R. W., Cold Spring Harbor Symposia on Quantitative Biology, Cold Spring Harbor, Long Island Biological Association, 1934, **2**, 39.
45. Yamaha, G., *Bot. Mag. Tokyo*, 1926, **40**, 172.

EXPLANATION OF PLATES

Plate 1

Figs. 1 and 2. Grasshopper spermatocytes in prophase, photographed at 2537 Å and 436 mμ. One uninjured and two injured cells. Zeiss 2.5 mm. quartz objective, 10 × quartz ocular. 900 × (2537 Å); 600 × (436 mμ).

Figs. 3 and 4. Same cells, after 20 minutes' irradiation with ultraviolet (2537 Å).

Figs. 5 and 6. Nucleus of onion epidermis cell, photographed at 2537 Å, living and fixed with acetic acid. Zeiss 2.5 mm. quartz objective, 10 × quartz ocular. 900 ×.

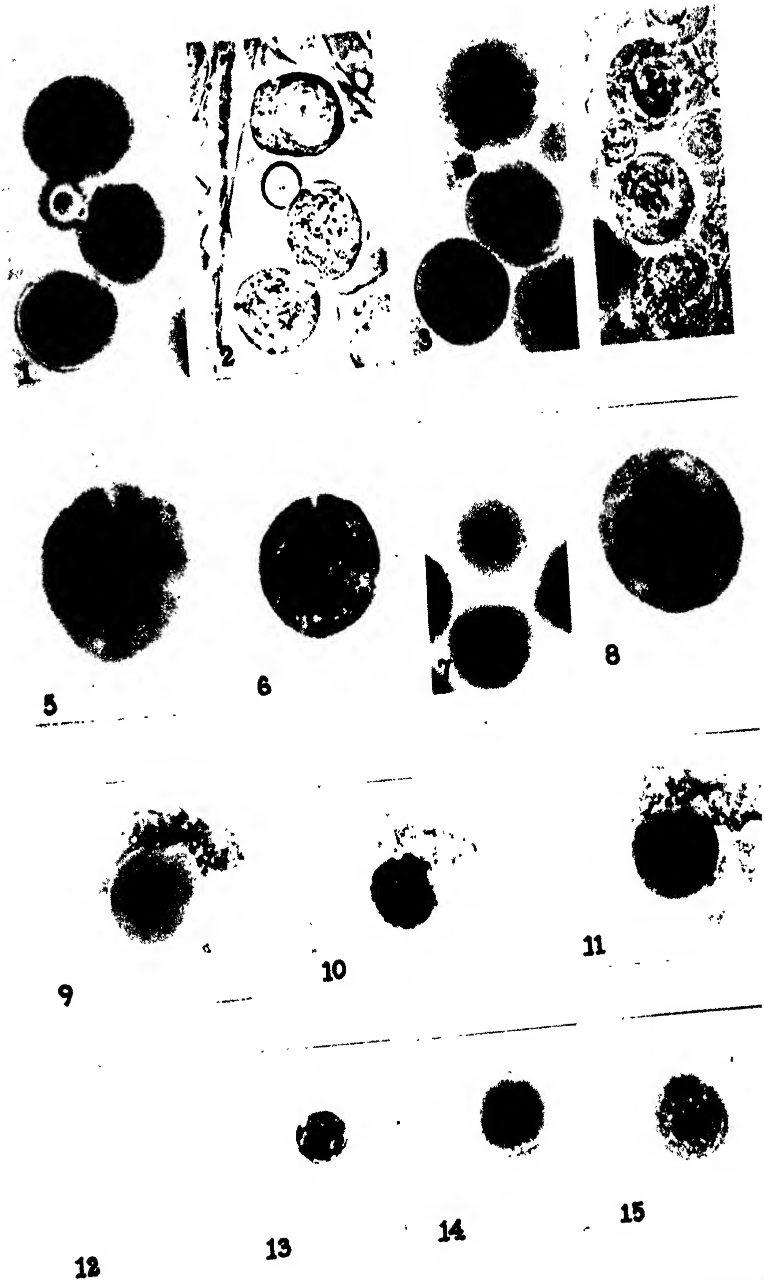
Fig. 7. Grasshopper spermatocyte prophase, killed with ultraviolet radiation (2537 Å) suspended in 10 per cent sucrose. 2537 Å, Zeiss 2.5 mm. quartz objective, 10 × quartz ocular. 900 ×.

Fig. 8. Onion epidermis nucleus, teased out in 10 per cent sucrose. 2537 Å, Zeiss 2.5 mm. quartz objective, 10 × quartz ocular. 900 ×.

Figs. 9, 10, and 11. Rat liver nucleus, teased free in 10 per cent sucrose (9); suspended in 0.8 per cent NaCl (10); salt washed out with 10 per cent sucrose (11). Reversible condensation of chromosomes in electrolyte solution. 2537 Å, Zeiss 2.5 mm. quartz objective, 10 × quartz ocular. 1800 ×.

Figs. 12 and 13. Calf thymus nuclei, suspended in 10 per cent sucrose and after fixation in Carnoy. 2537 Å, Zeiss 2.5 mm. quartz objective, 10 × quartz ocular. 1800 ×.

Figs. 14 and 15. Beef liver nuclei, suspended in 10 per cent sucrose and after fixation in Carnoy. 2537 Å, Zeiss 2.5 mm. quartz objective, 10 × quartz ocular. 1800 ×.



(Ris and Mirsky: State of chromosomes in interphase nucleus)

Plate 2

FIGS. 16 and 17. Isolated chromosomes from calf thymus, stained with methyl green in 0.8 per cent NaCl (16) and in 30 per cent sucrose (17). 630 mμ, Zeiss 2 mm. objective, 15 × ocular. 1200 ×.

FIG. 18. Onion epidermis nucleus, fixed in extended state with formalin and lanthanum acetate, stained with Feulgen. 546 mμ, Zeiss 2 mm. objective 15 × ocular. 1200 ×.

FIG. 19. Onion epidermis nucleus treated as in Fig. 18, but nucleus was in condensed state before fixation. 546 mμ, Zeiss 2 mm. objective, 15 × ocular. 1200 ×.

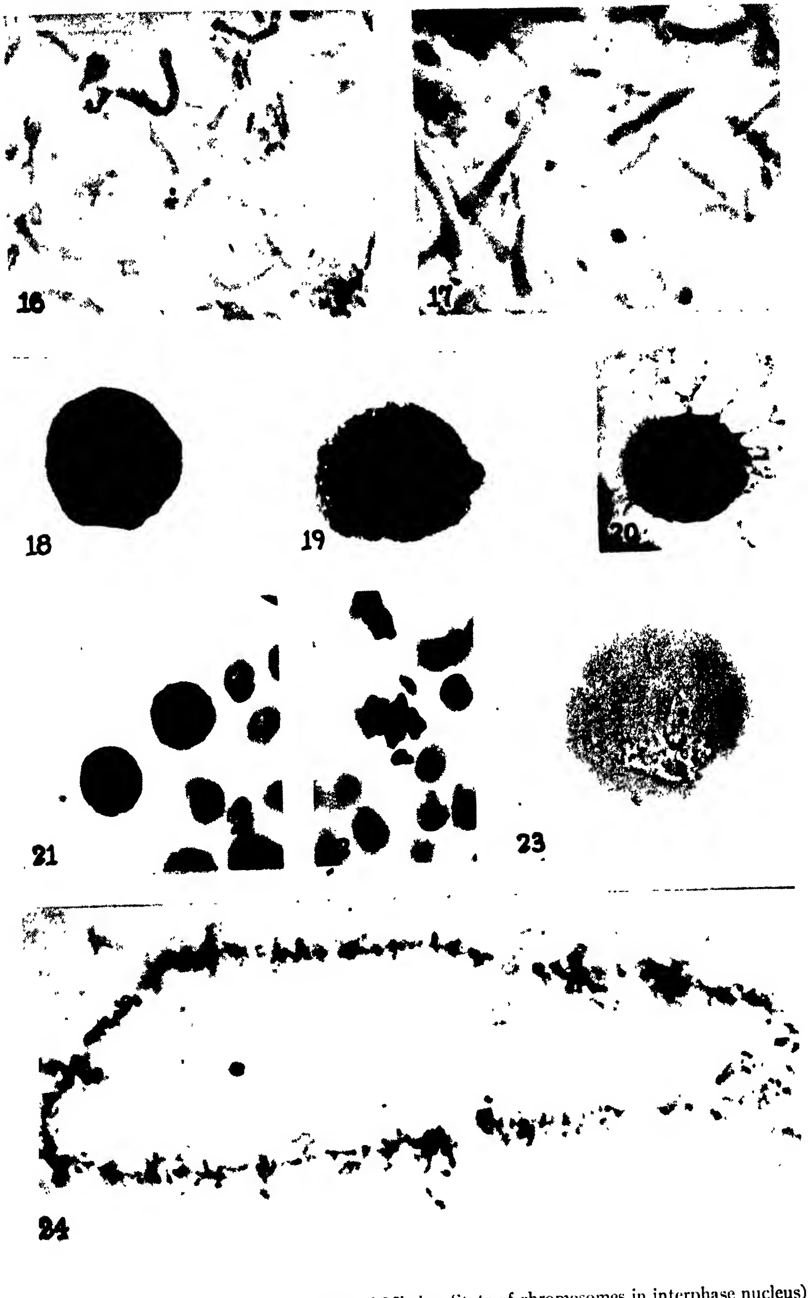
FIG. 20. Onion epidermis nucleus, fixed in extended state with formalin and lanthanum acetate, treated with trichloroacetic Millon to show distribution of protein. 365 mμ, Zeiss 2 mm. objective, 10 × ocular. 800 ×.

FIG. 21. Grasshopper spermatocyte prophases, fixed with formalin and lanthanum acetate, stained with Feulgen. 546 mμ, Zeiss 2 mm. objective, 15 × ocular. 1200 ×.

FIG. 22. Grasshopper spermatocyte, metaphase of first meiotic division. Fixed in formalin and lanthanum acetate, stained with Feulgen. 546 mμ, Zeiss 2 mm. objective, 15 × ocular. 1200 ×.

FIG. 23. *Drosophila pseudoobscura*, salivary gland nucleus teased out in 10 per cent sucrose, stained with methyl green 630 mμ, Zeiss 2 mm. objective, 15 × ocular. 1200 ×.

FIG. 24. Frog oocyte lampbrush chromosome. Germinal vesicle isolated in 10 per cent sucrose, stained with pyronin in sucrose. 546 mμ, Zeiss 2 mm. objective, 15 × ocular. 2400 ×.



(Ris and Mirsky: State of chromosomes in interphase nucleus)

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, June 1, 1949, Vol. 89, No. 6, pp. 681–686]

THE CYTOLOGY OF RICKETTSIAE

BY HANS RIS, PH.D., AND JOHN P. FOX, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research, and the International Health Division of The Rockefeller Foundation, New York*)

PLATES 35 AND 36

(Received for publication, March 4, 1949)

In recent years it has been shown that all bacterial cells which have been adequately examined are essentially similar to the cells of higher organisms with the demonstration of desoxyribonucleic acid-containing, regularly dividing nuclear structures and the presence of ribonucleic acid in the cytoplasm (1, 2). It is not yet clear whether these Feulgen-positive bodies are similar to chromosomes in higher organisms or whether the genic material is organized in a different way. Since chromosomes exhibit a very special structure and behavior during cell division in addition to containing DNA and being self-reproducing, this name should not be applied to the Feulgen-positive bodies of bacteria. Instead the less specific terms "nuclear structure" and "chromatinic body" (Robinow (1)) will be used here.

Rickettsiae are usually considered to be essentially like bacteria in morphology though they resemble viruses in being obligate intracellular parasites (*cf.* reference 3). Photographs with the electron microscope have revealed some internal structures similar to those found in bacteria (4). Chemical analysis of isolated rickettsiae, however, has shown the presence of desoxyribonucleic acid only, no ribonucleic acid having been detected (5, 6). The present study was undertaken in order to investigate, first, whether RNA can be demonstrated in unwashed rickettsiae using cytochemical methods, and secondly, whether the DNA is present in nuclear structures as in the bacteria above mentioned, or is diffusely distributed through the rickettsial bodies.

Materials and Methods

The material used in this study came from chick embryo yolk sacs infected with the Breinl strain of epidemic typhus (*Rickettsia prowazeki*). Yolk sac smears were air-dried, heat-fixed, and then immersed in Carnoy or in 20 per cent formalin. Concentrated suspensions of rickettsiae were obtained from yolk sac emulsions by repeated washing in saline.

Ultraviolet photographs (2537 Å) were obtained using a G.E. germicidal lamp (4 watt) with quartz-condensing lens, a Bäckström filter (20 per cent $NiSO_4$ plus 8.5 per cent $CoSO_4$ in distilled water), Zeiss 1.7 mm. quartz objective and Zeiss ×10 quartz ocular.

Rickettsiae for electron microscope photographs were extracted from yolk sacs, sulfate-precipitated, and inactivated with 1:5,000 merthiolate. A drop of this suspension was dried on formvar film, washed in distilled water to remove salts, and dried again for examination in the RCA Universal electron microscope.

Unstained smears of rickettsiae were also photographed with the phase contrast microscope (Spencer 1.8 mm., medium dark contrast objective).

To determine the presence of RNA in unwashed rickettsiae they were fixed in 20 per cent formalin and treated with ribonuclease (preparation of Dr. Kunitz, 0.2 mg. per ml. in distilled water, 45 minutes at 50°C.). Controls were treated the same way except for the enzyme. Buffer solutions were not used because they were found to extract the basophilic material from rickettsiae on the control slides. The slides were then stained together in methyl green pyronine for 20 minutes and differentiated in acetone.

Demonstration of Ribonucleic Acid in the Cytoplasm of Rickettsiae

Tovarnickij *et al.* (5) studied the chemical composition of rickettsiae isolated from mouse lungs and washed with physiological saline. Cohen (6) analyzed rickettsiae isolated from phenol-treated typhus vaccines. Both authors reported the presence of DNA, but no RNA was found. They concluded that rickettsiae were similar to viruses in containing only one type of nucleic acid, while bacteria and higher organisms always have both RNA and DNA. However, it has been shown that ribonucleoproteins are easily extracted from cells with physiological saline (7). It is therefore possible that no RNA was present in purified rickettsiae because it had been washed out during preparation. The presence of RNA in cells can be demonstrated cytochemically using ribonuclease and basic dyes (8). We therefore treated yolk sac smears fixed with 20 per cent formalin with ribonuclease and stained with methyl green pyronine. On the control slide the rickettsiae stain more or less solidly red with pyronine (Fig. 1). The intensity of the staining varies somewhat from one cell to the other. After digestion with ribonuclease, however, the over-all staining is always very much decreased (Fig. 2). Rickettsiae therefore contain RNA in variable amounts, probably depending on the physiological state as has been demonstrated for bacteria (9). Since it was not found in purified suspensions of rickettsiae it must have been lost during preparation. The effect of saline for instance on the staining with pyronine is marked. Fresh rickettsiae and rickettsiae washed with saline were smeared on the same slide and stained with pyronine. Unwashed rickettsiae stain uniformly red. Rickettsiae washed once stain very faintly and those washed more thoroughly do not stain at all with pyronine.

Recently Callot and Vendrely (10) studied the effect of desoxyribonuclease and ribonuclease on rickettsiae. They found that after desoxyribonuclease the staining with Giemsa was greatly reduced, but no marked decrease in staining was detected after digestion with ribonuclease. It is possible that the RNA was washed out during incubation in the control, or that they were dealing with rickettsiae in a physiological state with low RNA content in the cytoplasm.

Demonstration of Nuclear Structures in Rickettsiae

With the phase contrast microscope two or more dark bodies are visible in the rickettsial rods (Fig. 3). These structures are very similar to the chroma-

tinic bodies in bacteria. In order to determine whether they are nuclear structures like those in bacteria it must be shown that they contain DNA.

(*a*) *Staining with Basic Dyes.*—In bacteria the nuclear structures can be demonstrated with basic dyes after removal of the RNA of the cytoplasm. This is accomplished either with ribonuclease (11) or through hydrolysis with 1 N HCl (12). Robinow (13) hydrolyzes in 1 N HCl and then stains with Giemsa.

Rickettsiae in fresh yolk sac smears stain solidly with basic dyes such as basic fuchsin (Macchiavello's procedure) and pyronine. Rickettsiae which have been washed with saline before fixation lose the ability to stain with these dyes. If washed rickettsiae, or rickettsiae hydrolyzed with 1 N HCl at 60° for 10 minutes are stained with Giemsa chromatinic bodies become apparent (Fig. 4).

Methyl green is a basic dye with high specificity for DNA. Washed rickettsiae were stained with methyl green pyronine. The nuclear structures stained purplish and the cytoplasm faintly pink. Photographed at 630 mμ near the absorption maximum of methyl green, the nuclear structures were clearly visible. The chromatinic bodies, however, appeared most distinct after treatment with ribonuclease and staining with basic dyes. Fig. 2 shows rickettsiae stained with methyl green pyronine after ribonuclease treatment. The nuclear structures stained purplish and stand out clearly in the practically colorless cytoplasm. Fig. 6 is a photograph from the same slide, but taken with the phase contrast microscope.

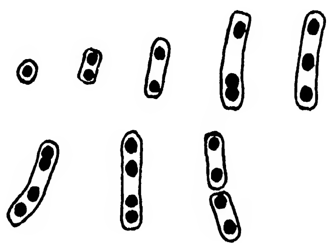
(*b*) *Ultraviolet Absorption.*—Photographs of washed rickettsiae at 2537 Å show strongly absorbing structures inside the rickettsial bodies (Fig. 5), corresponding to the structures staining with basic dyes. This is further evidence for the presence of nucleic acid in these structures.

(*c*) *Feulgen Reaction.*—Yolk sac smears were fixed in Carnoy and stained with the Feulgen reaction (modification of Rafalko (14)). The nuclear structures stained very faintly red. With a green filter (Wratten 74) the small dots of the chromatinic bodies could be seen, but nothing else of the rickettsiae was visible. Though the stain was so weak that by itself it would be questionable as a demonstration of DNA, it indicated that the DNA found in purified rickettsiae must be concentrated in these small structures inside the rickettsial bodies. The absolute amount of DNA in one rickettsial organism was obviously extremely small.

The behavior of these chromatinic bodies towards basic dyes, especially after digestion with ribonuclease, the absorption at 2537 Å, and the Feulgen staining therefore leave little doubt that the DNA found in rickettsiae is localized in definite nuclear structures. Spherical rickettsiae contain one nuclear body. In rod-shaped rickettsiae one finds two bodies which are close together in short rods and farther separated in long rods. Sometimes long rods may contain

three or four chromatinic bodies. These are usually spherical, but occasionally one sees dumbbell-shaped structures which suggest a chromatinic body in the process of division (Text-fig. 1, and Figs. 6 and 9).

Electron microscope photographs of rickettsiae washed with saline revealed internal structures which correspond to the chromatinic bodies described above.[1] Rickettsiae with one, two, or three chromatinic bodies were common (Figs. 7 to 12). Sometimes two nuclear structures were very close together, possibly representing the division of a chromatinic body (Figs. 9 and 10). Plotz *et al.* (4) described structures which seem to be identical with our chromatinic bodies.



TEXT-FIG. 1. Nuclear structures in various forms of *Rickettsia prowazeki*. Compare with Figs. 6 and 7 to 12.

SUMMARY

Internal structures of rickettsiae seen with phase contrast microscopy and in the electron microscope contain desoxyribonucleic acid and are therefore nuclear structures similar to those found in bacteria. They are minute spherical bodies, either single as in spherical rickettsiae or varying in number from 2 to 4 in rod-shaped forms. Occasional dumbbell-shaped chromatinic bodies are thought to represent these structures in the process of division. The presence of ribonucleic acid in the cytoplasm of rickettsiae was demonstrated with the use of ribonuclease and basic dyes. Rickettsiae therefore have a cellular organization similar to that of certain bacteria, with a clear differentiation into nuclear structure and cytoplasm.

BIBLIOGRAPHY

1. Robinow, C. F., Addendum in Dubos, R. J., The Bacterial Cell, The Harvard University Press, Cambridge, 1945.
2. Boivin, A., in Cold Spring Harbor Symposia on Quantitative Biology, Cold Spring Harbor, Long Island Biological Association, 1947, **12**, 7.
3. Pinkerton, H., *Bact. Rev.*, 1942, **6**, 37.
4. Plotz, H., Smadel, J. E., Anderson, T. F., and Chambers, L. A., *J. Exp. Med.*, 1943, **77**, 355.

[1] The electron microscope photographs were prepared by Dr. E. G. Pickels, formerly at the Rockefeller Institute.

5. Tovarnickij, V. I., Krontovskaja, M. K., and Ceburkina, N. V., *Nature*, 1946, **158**, 912.
6. Cohen, S. S., *Fed. Proc.*, 1946, **5**, 129.
7. Mirsky, A. E., and Pollister, A. W., *J. Gen. Physiol.*, 1946, **30**, 117.
8. Brachet, J., *Compt. rend. Soc. biol.*, 1940, **133**, 88.
9. Caspersson, T., Malmgren, B., Thorell, B., and Bjerkelund, E., *Nord. Med.*, 1945, **28**, 2636.
10. Callot, J., and Vendrely, R., *Compt. rend. Soc. biol.*, 1948, **142**, 396.
11. Tulasne, R., and Vendrely, R., *Compt. rend. Soc. biol.*, 1947, **141**, 674.
12. Vendrely, R., and Lipardy, J., *Compt. rend. Acad. sc.*, 1946, **223**, 342.
13. Robinow, C. F., *Proc. Roy. Soc. London, Series B*, 1942, **130**, 299.
14. Rafalko, J. S., *Stain Technol.*, 1946, **21**, 91.

EXPLANATION OF PLATES

PLATE 35

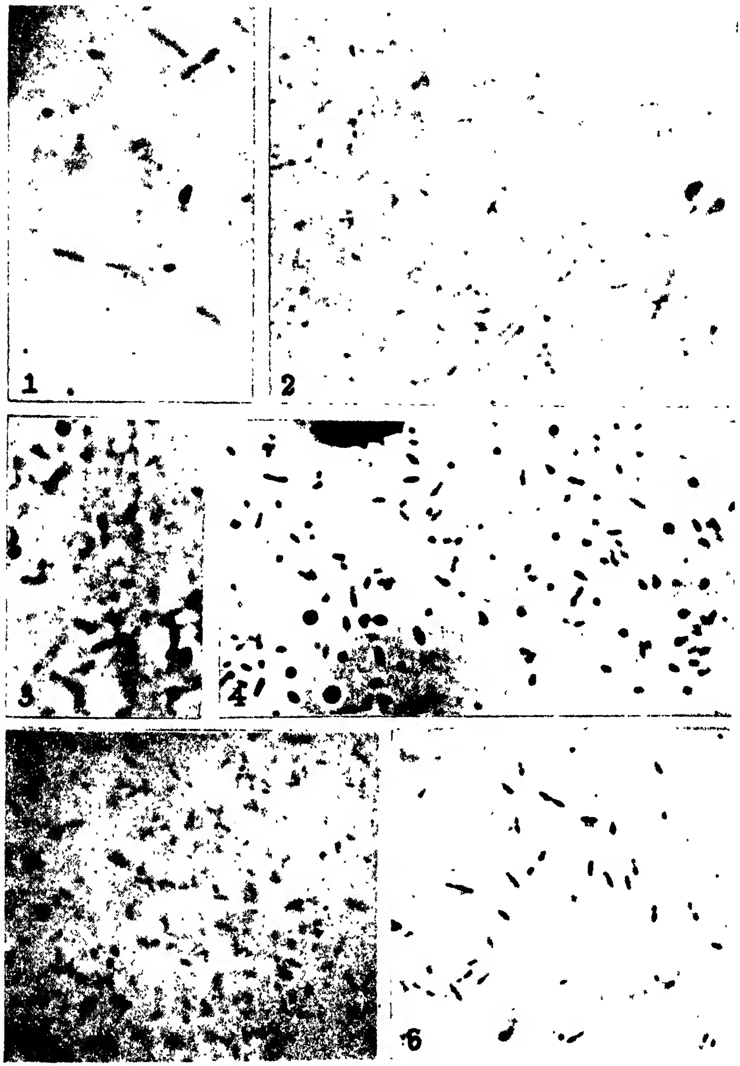
FIGS. 1 and 2. Rickettsiae in yolk sac smears, stained with methyl green pyronine. Fig. 2 shows the rickettsiae after treatment with ribonuclease, Fig. 1 in the control slide. In the control the cytoplasm is stained intensely with pyronine. After ribonuclease treatment only the nuclear structures are stained. Zeiss 2 mm. NA 1.3 objective, × 2400.

FIG. 3. Photograph of unwashed rickettsiae in yolk sac smear taken with the phase contrast microscope. Spencer 1.8 mm. dark medium, × 2700.

FIG. 4. Rickettsiae in yolk sac smear, hydrolyzed with N HCl 10 minutes, stained with Giemsa. Zeiss 2 mm. NA 1.3 objective, × 2400.

FIG. 5. Rickettsiae washed with saline, photographed in ultraviolet light (2537 Å), Zeiss 1.7 mm. quartz objective, *ca.* × 2500. The nuclear structures absorb more intensely than the cytoplasm.

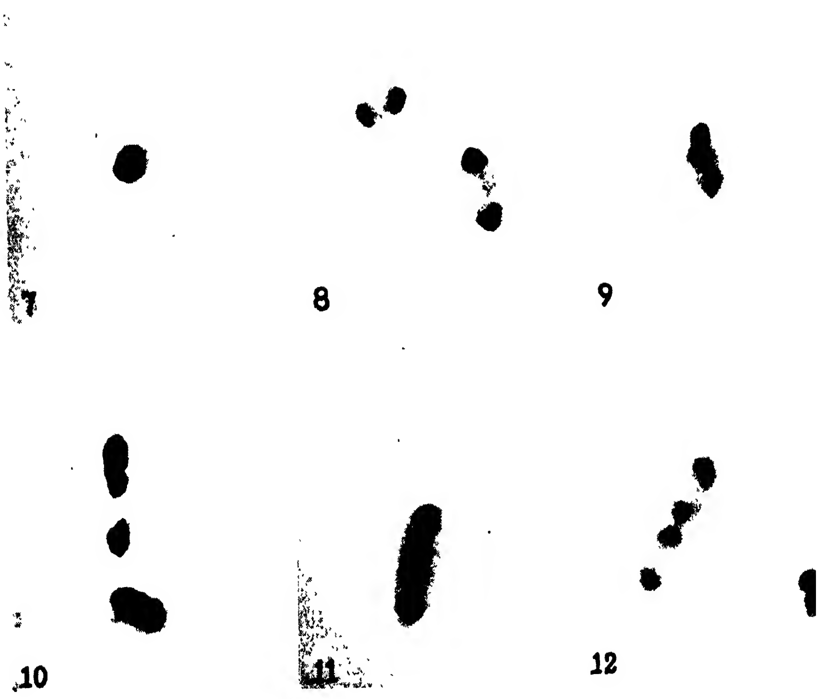
FIG. 6. Rickettsiae in yolk sac smear, digested with ribonuclease, stained with methyl green pyronine, photographed with the phase contrast microscope. Spencer 1.8 mm. dark medium objective, × 2700. The chromatinic bodies stand out most sharply with this technique.



(Ris and Fox: Cytology of rickettsiae)

PLATE 36

FIGS. 7 to 12. Electron microscope photographs of rickettsiae extracted from yolk sacs, sulfate-precipitated, and inactivated with merthiolate. RCA Universal electron microscope, *ca.* × 15,000. Compare with Fig. 6.



(Ris and Fox: Cytology of rickettsiae)

[Reprinted from THE JOURNAL OF GENERAL PHYSIOLOGY, March 20, 1949, Vol. 32, No. 4, pp. 553–557]

MOVEMENTS OF WATER IN CELLS OF NITELLA

BY W. J. V. OSTERHOUT

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, November 5, 1948)

The behavior of water in the living cell is highly important but our knowledge regarding it is very unsatisfactory. Since cells of *Nitella* are very favorable for such study some experiments have been made some of which are reported here.

They deal with the forces which cause water to enter and to leave the cell and show how such movements of water can be quantitatively predicted.

Since the cells show little or no permeability to sucrose the experiments have been made with solutions of sucrose in which the cell behaves as a fairly good osmometer.

Movements of water can be induced in cells of *Nitella* by placing water at one end, A, and a solution of sucrose at the other, B. Water then enters at A and passes to B where it escapes. Solutes in the cell move with the water from A to B but do not escape at B since they are unable to pass out through the protoplasm except very slowly. As a result the internal osmotic pressure decreases at A and increases at B.

The movement of water is due to the osmotic drive which forces water into the cell; this may be defined as the difference between the internal and the external osmotic pressure.

If the osmotic drive at A is greater than at B water moves from A to B. The osmotic drive falls off at A and increases at B until the two values become equal. The motion then stops.

Experiments have been made to determine the final equilibria attained and an equation has been obtained which enables us to predict the results. The agreement between prediction and observation is fairly satisfactory.

EXPERIMENTAL

The *Nitella* cell has a layer of protoplasm not over 15 microns in thickness surrounding a large central vacuole filled with sap (this is over 450 microns in diameter). Outside this is a cellulose wall about 15 microns thick.[1]

[1] The observations were made on *Nitella flexilis*, Ag. The cells were freed from neighboring cells and observed at once or kept in the laboratory in Solution A (*cf.* Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933–34, **17,** 87) at 15°C. ± 1°C. About 15 hours before use the cells were placed in a large amount of Solution A

The movement of water can easily be observed under the microscope if the *Nitella* cell[2] is placed on a slide with a barrier of vaseline in the center to prevent the water at A from mixing with the solution at B. The rush of water from A to B is easily visible because it carries with it particles suspended in the cell sap.

If the sap is stained with brilliant cresyl blue[3] we see that the movement of water carries the dye with it so that the color becomes paler at A and deeper at B.

For quantitative work we may employ the method described below.

Cells 5 to 8 cm. long were placed in the apparatus shown in Fig. 1. A cork 1.7 cm. long and 8 mm. in diameter was split lengthwise by a sharp razor and a shallow groove was made in one half. A *Nitella* cell was placed in this surrounded by vaseline to make a water-tight seal and the whole of the flat surface of the cork was covered with vaseline. A glass tube R completely filled with water was then fitted over the right end and at the left end a tube L filled with water was fitted on; this tube ended in a calibrated capillary with scale divisions etched in the glass (the capillary was 15 cm. long). Care was taken to exclude air bubbles from both tubes.[4]

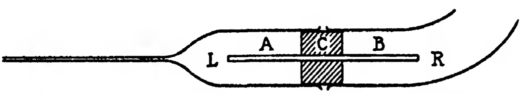


FIG. 1. Apparatus for measuring the movement of water in cells of *Nitella*. The cell is held under gentle pressure in the cork, C. The tubes L and R are filled with water and the apparatus is left until the meniscus in the capillary of L becomes stationary. Water in R is then replaced by a solution of sucrose. We then find that water enters the cell at A, passes along inside the cell, and escapes at B.

The diameter of the cork was such that the tubes compressed the cork enough to make a water-tight seal without injuring the cell. The seal was tested by tipping the apparatus to make sure that no movement of water occurred under the influence of gravitation. In use the apparatus was kept horizontal and was supported on a strip of wood in a groove of the right size to hold it firmly.

If the apparatus has been set up properly the meniscus at the end of the capillary will be stationary and not easily affected by adding or subtracting liquid at R.

At the start L and R were filled with water and the meniscus at the end of L was

---

in a room at about 25°C. and the temperature of the solution rose gradually to about 25°C. and the experiments were performed at about this temperature.

Microscopic observations show that the dimensions of the cell do not change during the experiments so that we may conclude that when a given amount of water enters at one end the same amount escapes at the other end.

The use of metal forceps was avoided.

[2] The movement of particles in the sap of the vacuole may occur without disturbing the normal protoplasmic movement (cyclosis). *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1947, **30,** 439.

[3] Irwin, M., *J. Gen. Physiol.*, 1926, **9,** 561; 1926, **10,** 75.

[4] If necessary the fitting of the tubes to the cork may be done with the cork submerged in water or in solution.

observed to see when it became steady. If this took longer than 10 minutes a new cell was introduced into the apparatus. When the meniscus had become steady the apparatus was tipped to make sure that there was a good seal at the cork in which case the meniscus did not move; if the seal was defective this was corrected.

The water in R was then removed by means of a pipette with as little mechanical disturbance as possible and a new solution was introduced into R.

The movement of liquid in the capillary was observed under a magnifying glass and the time was read on a stop watch (if necessary one observer watched the capillary and another the stop watch).

Microscopic observations of cells with sap containing dye showed that with water at A and sucrose solution at B any backward diffusion from B to A was negligible.

After each experiment the cells were kept under observation for 2 days. Unless they remained in good condition the experiment was rejected. Further observation does not appear to be necessary since if no signs of injury appear in 2 days they usually live indefinitely. The condition of the cell was judged by its turgor and its microscopic appearance.

All osmotic pressures are recorded at 25°C.

Equilibrium Values

If we know the internal and external osmotic pressure and the volumes of A and B we can predict the total amount of flow and the osmotic pressures at equilibrium when the osmotic drive at A (or D_A) becomes equal to that at B (or D_B). This final value when $D_A = D_B$ may be called D_F.

To illustrate the calculation of D_F we may consider a case where the combined lengths of A and of the cork amount to 5 cm. and they are both regarded as A because they act alike in giving up solute to B. We assume for convenience in calculation that the volume of A (or V_A) is 5 and that of B (or V_B) is 1.

We assume that the osmotic pressure in the cell is due to a single solute, S, and that in each section 1 cm. in length there are[5] 6.4 x mols of S giving an osmotic pressure of 6.4 atmospheres (all values relate to 25°C.).[6] We assume that S cannot pass out of the protoplasm.

If we put water at A and a solution of sucrose with an osmotic pressure of 6 atmospheres at B we have the following situation:

At A	At B
$P_{IA} = 6.4$	$P_{IB} = 6.4$
$P_{OA} = 0$	$P_{OB} = 6$
$D_A = 6.4 - 0 = 6.4$	$D_B = 6.4 - 6 = 0.4$

Here P_I is the internal and P_O the external osmotic pressure and D the osmotic drive. Since D_A is greater than D_B water enters at A, moves along inside the

[5] The value of x might be of the order of 10^{-7}. It is assumed that the value of x is constant for each cell but that it may vary from cell to cell.

[6] The value 6.4 was arrived at by plasmolytic experiments.

cell, and escapes at B. Since S is unable to pass out through the protoplasm it cannot escape from the cell and its concentration increases at B while it diminishes at A. Hence D_A becomes less and D_B increases until they become equal so that $D_A = D_B = D_F$.

Let us now consider the excess of osmotic drive at A as compared with B. If we remove this excess the osmotic drive will become equal throughout the cell. The excess is $D_A - D_B = 6.4 - 0.4 = 6$. To remove this we must take $6\,x$ mols of S from each of the 5 sections of A. Hence the amount removed is $V_A(D_A - D_B) = 5\,(6.4 - 0.4) = 30\,x$ mols of S. The osmotic drive is now 0.4 everywhere and if we distribute the $30\,x$ mols uniformly throughout the cell the osmotic drive will remain equal everywhere. For this purpose we divide the $30\,x$ mols into 6 equal parts and give 1 part to each section. Since the volume of the cell, or V_{cell}, is 6 we may write for the amount each section will receive:

$$\frac{V_A(D_A - D_B)}{V_{cell}} = \frac{5(6.4 - 0.4)}{6} = 5x \text{ mols}$$

When this is added to D_B we have:

$$\frac{V_A(D_A - D_B)}{V_{cell}} + D_B = \frac{5(6.4 - 0.4)}{6} + 0.4 = 5.4$$

This is now the value of the osmotic drive at all points and hence it is the value of D_F. We therefore have:

At A	At B
$P_{IA} = 6.4 - 6 + 5 = 5.4$	$P_{IB} = 6.4 + 5 = 11.4$
$P_{OA} = 0$	$P_{OB} = 6$
$D_A = 5.4 - 0 = 5.4$	$D_B = 11.4 - 6 = 5.4$

Since $D_A = D_B$ the motion stops. The value of D_F is 5.4.

The agreement between calculation and observation was tested in the following manner. Cells were selected in which the diameter of the vacuole (in which the water chiefly moves) was approximately the same as the bore of the capillary (473 microns). If the length[7] of A is 3 cm. and the motion in the capillary from left to right is 2 cm. it is evident that 66.7 per cent of the liquid in A has moved to B. The cells in which the diameter of the vacuole was approximately equal to the bore of the capillary consisted of 2 lots. In the first lot of 4 cells the ratio $V_A \div V_{cell}$ in each cell was equal to 0.604. Water was placed at A and 0.4 M sucrose with an osmotic pressure of 11.2 atmospheres at B. The value of D_B was -4.8 hence that of D_F was 1.96 and the calculated loss at A was 100 $(6.4 - D_F) \div 6.4$ or 100 $(6.4 - 1.96) \div 6.4 = 69.4$ per cent. The average observed value as shown by the flow was 66.9 per cent.

In another lot of 7 such cells with a ratio of $V_A \div V_{cell}$ of approximately 0.548

[7] This includes the area under the cork since this acts like A in giving up solute to B.

in each cell water was placed at A and 0.2 M sucrose with an osmotic pressure of 5.1 atmospheres at B. The average calculated value of the loss at A was 36.0 per cent and the observed value 35.3 per cent (Standard deviation 7.2).

Calculations of D_F show that the total volume of flow required to produce equilibrium if expressed as per cent of the volume of the cell is at a maximum when $V_A \div V_B = 1$ and falls off regularly as the value of this ratio increases or diminishes. This agrees in general with observation but there is considerable variability.

These observations show that the average amount of flow needed to produce equilibrium can be predicted with considerable accuracy. In making the calculations we assume that the movement of solute corresponds to the movement of liquid so that when half of the liquid in A moves to B half of the solute also moves. If not all the solute were equally affected by the flow the movement would exceed the predicted amount. But if the flow were stopped by the aggregation of colloidal masses in the sap before true equilibrium occurred the amount would be less than the calculated value.

The kinetics of flow present some interesting features which may be taken up In a later paper.

I wish to thank Mr. Jerome S. Fass for the care and skill he has shown in carrying out these experiments.

SUMMARY

When one end of a *Nitella* cell (A) is bathed in water and a solution of sucrose is placed at the other (B) we find that water enters at A, travels along inside the cell, and escapes at B. The solutes which cannot pass out through the protoplasm at B remain behind so that the osmotic pressure increases at B and diminishes at A until equilibrium is reached and the motion stops.

An equation is given which enables us to predict with considerable accuracy the amount of flow required to produce equilibrium.

[Reprinted from THE JOURNAL OF GENERAL PHYSIOLOGY, March 20, 1949,
Vol. 32, No. 4, pp. 559–566]

TRANSPORT OF WATER FROM CONCENTRATED TO DILUTE SOLUTIONS IN CELLS OF NITELLA

BY W. J. V. OSTERHOUT

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, November 5, 1948)

The transport of water from concentrated to dilute solutions which occurs in the kidney and in a variety of living cells presents a problem of fundamental importance.

If the cell acts as an osmometer we may expect to bring about such transport by creating an inwardly directed osmotic drive which is higher in one part of the cell than in other regions of the same cell. The osmotic drive is defined as the difference between internal and external osmotic pressure.

Experiments with *Nitella* show that this expectation is justified. If water is placed at one end of the cell (A) and 0.4 M sucrose with an osmotic pressure of 11.2 atmospheres at the other end (B) water enters at A, passes along inside the cell, and escapes at B leaving behind at B the solutes which cannot pass out through the protoplasm. Hence the internal osmotic pressure becomes much higher at B than at A. When 0.4 M sucrose at B is replaced by 0.3 M sucrose with an osmotic pressure of 8.1 atmospheres we find that water enters at B, passes along inside the cell, and escapes at A so that water is transported from a concentrated to a dilute solution although the difference in osmotic pressure of the 2 solutions is more than 8 atmospheres. The solution at B thus becomes still more concentrated.

EXPERIMENTS

Cells of *Nitella*[1] 5 to 8 cm. long were employed. In these cells the protoplasm forms a layer not over 15 microns thick surrounding a large central vacuole over 450 microns in diameter; outside the protoplasm is a cellulose wall about 15 microns thick.

The cells were placed in the apparatus described in a previous paper[2] (see Fig. 1). The center of the *Nitella* cell was held under gentle pressure in a piece of cork 1.7 cm. long so as to make a water-tight seal. At the right end (B) the cell was surrounded

[1] The observations were made on *Nitella flexilis*, Ag. The cells were freed from neighboring cells and observed at once or kept in the laboratory in Solution A (*cf.* Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933–34, 17, 87) at 15°C. ± 1°C. About 15 hours before use the solution containing the cells was allowed to warm up slowly to about 25°C. and the experiments were performed at about this temperature. All osmotic pressures are taken at 25°C.

[2] Osterhout, W. J. V., *J. Gen. Physiol.*, 1949, **32**, 553.

by liquid in the tube R; at the left end (A) it was surrounded by water in the tube, L, ending in a calibrated capillary so that any movement of water in the tube could be measured by observing the movement of the meniscus in the capillary.

All the precautions described in the previous paper[2] were observed.

When water was placed at A and 0.4 M sucrose at B, water entered at A, passed along inside the cell, and escaped at B. This motion of water is due to the osmotic drive which forces water into the cell at A.

The internal osmotic pressure as judged by plasmolytic experiments with sucrose is about 6.4 atmospheres at 25°C. (all osmotic pressures are given at this temperature). Hence when we place water at A the osmotic drive tending to force water into the cell is $6.4 - 0 = 6.4$ atmospheres. Since the same situation exists at B the forces are equal and opposite and there is no motion of water. When we place water at A and sucrose solution at B the osmotic drive is greater at A and water enters at A, passes along inside the cell, and escapes at B. The water carries solutes from A to B which are unable to escape at B because they cannot pass out through the protoplasm. Hence the concentration of solutes and consequently the osmotic drive

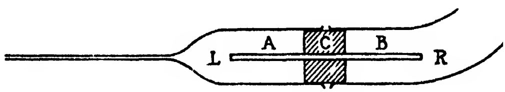


FIG. 1. Apparatus for measuring the movement of water in cells of *Nitella*. The cell is held under gentle pressure in the cork, C. The tubes L and R are filled with water and the apparatus is left until the meniscus in the capillary of L becomes stationary. Water in R is then replaced by a solution of sucrose. We then find that water enters the cell at A, passes along inside the cell, and escapes at B.

falls off at A and increases at B until the osmotic drive becomes equal at both places. The motion then stops.

Experiments made by placing water at A and 0.4 M sucrose with an osmotic pressure of 11.2 atmospheres at B gave 20 mm. as the average amount of flow in the capillary from A to B. The 0.4 M sucrose at B was then replaced by 0.3 M sucrose, with an osmotic pressure of 8.1 atmospheres. Water then entered at B, passed along inside the cell, and escaped at A (Fig. 2). The cell therefore transferred water from 0.3 M sucrose at B with an osmotic pressure of 8.1 atmospheres to water at A. The water thus transferred escaped into the water surrounding the cell at A.

This behavior of water can be demonstrated by placing the cell on a microscope slide with a barrier of vaseline in the center to keep the sucrose solution at B from mixing with the water at A. Observing the cell under the microscope we see that as water enters the cell at A and moves to B there is a rapid movement from A to B of particles suspended in the sap of the vacuole.[3] If the sap is stained with brilliant cresyl blue[3] the dye moves from A to B and becomes paler in color at A and deeper at B since it does not escape through the protoplasm.

When the 0.4 M sucrose at B is replaced by 0.3 M sucrose there is a rapid movement

---

[3] The movement of particles in the sap of the vacuole may take place while the normal protoplasmic motion continues. Regarding the dye see Irwin, M., *J. Gen. Physiol.*, 1926, **9**, 561; 1926, **10**, 75.

of particles in the sap from B to A and if dye is present it becomes paler in color at B and deeper at A. The experiment may be varied by removing the water surrounding A and replacing it by mineral oil[4] leaving only a film of water adhering to the cellulose wall. When water moves from B to A we see drops of water emerging into the oil at A.

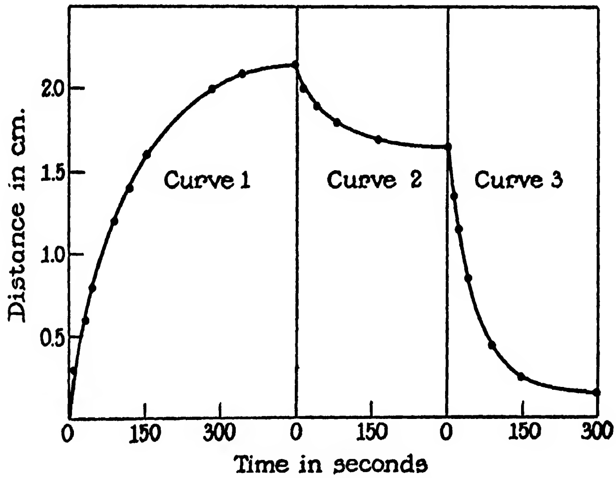


FIG. 2. Time curves of the flow of water in a typical experiment. Curve 1 shows the flow from A to B with water at A and 0.4 M sucrose at B.

Curve 2 shows the flow from B to A when 0.4 M sucrose at B is replaced by 0.3 M sucrose; this flow carries water from the external solution of 0.3 M sucrose with an osmotic pressure of 8.1 atmospheres at B to external water at A, thus increasing the concentration of the sucrose solution at B.

Curve 3 shows the flow from B to A when 0.3 M sucrose at B is replaced by water. This carries water from the external water at B to the external water at A.

In curves 2 and 3 the ordinates should be read downwards since the flow in the capillary is from right to left.

The length of A including the area under the cork (1.7 cm.) was 2.9 cm. and that of B was 1.8 cm.

When the 0.3 M sucrose at B was replaced by water there was a flow in the capillary from right to left and water escaped at A (Fig. 2). This was confirmed by microscopic observation.

Calculations

Microscopic measurements show no change in the dimensions of the cells during the experiments so that we may assume that when a given amount of water leaves the cell at A the same amount enters at B.

[4] Heavy mineral oil for medicinal use may be used. *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1947, **30**, 439.

We may assume for convenience that each section of the cell having a length of 1 cm. contains 6.4 x mols of a solute, S, giving an osmotic pressure of 6.4 atmospheres at 25°C. We assume that this is the only solute present and that it cannot pass out through the protoplasm.

When we place water at A and 0.4 M sucrose with an osmotic pressure of 11.2 atmospheres at B we have the following situation:

At A	At B
$P_{IA} = 6.4$	$P_{IB} = 6.4$
$P_{OA} = 0$	$P_{OB} = 11.2$
$D_A = 6.4 - 0 = 6.4$	$D_B = 6.4 - 11.2 = -4.8$

Here P_I is the internal and P_O the external osmotic pressure and D is the osmotic drive.

Since D_A is greater than D_B water enters at A, passes along inside the cell, and escapes at B leaving behind the solutes in the sap which are unable to pass out through the protoplasm. Hence the osmotic drive decreases at A and increases at B until both values become equal and the motion stops. The final value when $D_A = D_B$ may be called D_F. This may be calculated by means of the equation given in a former paper:[2]

$$D_F = \frac{V_A(D_A - D_B)}{V_{cell}} + D_B$$

Here V_A is the volume of A and V_{cell} the volume of the cell.

Let us consider a case where $V_A \div V_B = 1$. The area under the cork is regarded as part of A since it acts like A in giving up water to B. Since only relative volumes are required we may for convenience put $V_A = 1$ cm., V_B 1 cm., and V_{cell} 2 cm.

Substituting numerical values we have:

$$D_F = \frac{1(6.4 - (-4.8))}{2} + (-4.8)$$
$$= 0.8$$

Hence when the motion stops we have:

At A	At B
$P_{IA} = 6.4 - 5.6 = 0.8$	$P_{IB} = 6.4 + 5.6 = 12.0$
$P_{OA} = 0$	$P_{OB} = 11.2$
$D_A = 0.8 - 0 = 0.8$	$D_B = 12.0 - 11.2 = 0.8$

This means that from A with a length of 1 cm. 5.6 x mols of S have moved over to B thereby lowering the osmotic pressure at A from 6.4 to $6.4 - 5.6 = 0.8$. When this is added to the 6.4 x mols of S already present in B we have 12.0 x mols in a section 1 cm. long and consequently the osmotic pressure is 12.0 atmospheres. The loss at A is $(5.6 \div 6.4)\ 100 = 87.5$ per cent.

If we now replace the 0.4 M sucrose at B by 0.3 M sucrose having an osmotic pressure of 8.1 atmospheres we have:

At A	At B
$P_{IA} = 0.8$	$P_{IB} = 12.0$
$P_{OA} = 0$	$P_{OB} = 8.1$
$D_A = 0.8 - 0 = 0.8$	$D_B = 3.9$

As D_B is greater than D_A water enters at B, passes along inside the cell, and escapes at A leaving behind the solutes which cannot pass out through the protoplasm. Hence the osmotic pressure falls off at B and increases at A until the motion stops. We then have $D_A = D_B = D_F$. We may calculate the value of D_F as follows. Since the motion is from B to A we write:[5]

$$D_F = \frac{V_B(D_B - D_A)}{V_{cell}} + D_A$$

$$= \frac{1(3.9 - 0.8)}{2} + 0.8$$

$$= 2.35$$

We then have:

At A	At B
$P_{IA} = 0.8 + 1.55 = 2.35$	$P_{IB} = 12.0 - 1.55 = 10.45$
$P_{OA} = 0$	$P_{OB} = 8.1$
$D_A = 2.35 - 0 = 2.35$	$D_B = 10.45 - 8.1 = 2.35$

This means that 1.55 x mols of S have moved from B to A raising its internal osmotic pressure from 0.8 to 2.35 atmospheres.

The loss at B is $100\ (1.55 \div 12.0) = 12.9$ per cent.

If the 0.3 M sucrose at B is replaced by water we have the following situation:

At A	At B
$P_{IA} = 2.35$	$P_{IB} = 10.45$
$P_{OA} = 0$	$P_{OB} = 0$
$D_A = 2.35 - 0 = 2.35$	$D_B = 10.45 - 0 = 10.45$

Since D_B is greater than D_A water moves from B to A. We may calculate the value of D_F as before:

$$D_F = \frac{1(10.45 - 2.35)}{2} + 2.35$$

$$= 6.4\,.$$

[5] In the forward movement from A to B the area under the cork is regarded as part of A since it acts like A in giving up solute to B. In the backward movement from B to A the area under the cork is regarded as part of A since it acts like A in receiving solute from B.

We then have:

At A	At B
$P_{IA} = 2.35 + 4.05 = 6.4$	$P_{IB} = 10.45 - 4.05 = 6.4$
$P_{OA} = 0$	$P_{OB} = 0$
$D_A = 6.4 - 0 = 6.4$	$D_B = 6.4 - 0 = 6.4$

This means that 4.05 x mols of S have moved from B to A raising its internal osmotic pressure from 2.35 to 6.4 atmospheres.

The fact that we arrive at the value 6.4 for D_F shows that the method of calculation is correct since this was the value at the start.

The loss at B is $(4.05 \div 10.45)\ 100 = 38.8$ per cent.

The total loss of S at B in 2 steps is $1.55 + 4.05 = 5.6$ x mols which is the amount moving from A to B.

The cell is now restored to its original state at the start of the experiments. It is in contact with water at A and B and has 6.4 atmospheres of pressure at all points.

The agreement between calculation and observation was tested by selecting cells in which the diameter of the vacuole (in which the liquid chiefly moves) was approximately the same as the bore of the capillary (473 microns). Then if A is 3 cm. long it is evident that a movement of 2 cm. in the capillary from A to B means a loss of 66.7 per cent at A. A lot of 4 cells was used in each of which the value[6] of $V_A \div V_{cell}$ was 0.604. With water at A and 0.4 M sucrose with an osmotic pressure of 11.2 atmospheres at B we have $D_A = 6.4$, $D_B = -4.8$, and $D_F = 1.96$. The calculated loss at A is $100\ (6.4 - 1.96) \div 6.4 = 69.4$ per cent. The average observed loss was 66.9 per cent. Another lot containing 10 such cells in each of which the value of $V_A \div V_{cell}$ was approximately 0.599 gave for the average calculated loss at A 70.1 per cent and for the average observed loss 71.4 per cent.[7]

In the backward movement from B to A when 0.4 M sucrose is replaced by 0.3 M sucrose and then by water (Fig. 2) we might expect less flow than in the forward movement. Although the amount of solute which moves backward is the same as that which moves forward the concentration of the sap is higher in the backward movement. The backward flow is often less than the forward flow (Fig. 2) but as a rule it is greater than the predicted amount. This may be due to a greater dilution of the sap by the incoming water at B.

Returning to the hypothetical case where $V_A = 1$ cm. and $V_B = 1$ cm. we may say that if the forward flow is 87.5 per cent of 1 cm. or 0.875 cm. (page 450) we should expect the backward flow when 0.4 M sucrose is replaced by 0.3 M sucrose to be 0.129 cm. (page 451) and the backward flow when 0.3 M

[6] Here V_A includes the area under the cork since it acts like A in giving up solute to B.

[7] The standard deviation is 16.8.

sucrose is replaced by water to be 0.388 cm. (page 452) making a total of 0.517 cm. as compared with the forward flow of 0.875 cm.

We cannot expect close agreement in all cases between calculation and observation since there are variables which cannot be controlled. The calculation assumes a close correspondence between the movement of liquid and the movement of solute so that if half the liquid moves from A to B half the solute moves also. But if the entering water does not affect all of the solute equally the flow of water may be greater than expected. Or if the flow is stopped by the accumulation of colloidal masses in the vacuole so as to produce a stoppage before equilibrium is attained the flow will be less than expected.

DISCUSSION

The experiments show that water may move from a concentrated to a dilute solution when the osmotic drive is greater in one part of the cell than in other regions. Such a condition could doubtless arise if metabolism were not uniform throughout the cell and this would be favored if different regions of the cell were in contact with different external situations.

To maintain a flow of water from a concentrated solution at B to a dilute solution at A by metabolism it would be necessary to produce osmotically active substance inside the cell at B which would escape at A or become osmotically less active at A. Such a process might be periodic rather than continuous and during the periods when no transport from concentrated to dilute solutions occurred the solutes in the cell might alter so as to make such transport possible in a subsequent period.

If a substance M at B were converted to a substance N with a lower molecular weight the internal osmotic pressure at B would increase and water might move from B to A even if the external osmotic pressure were higher at B than at A. If N moved with the water inside the cell from B to A and there became polymerized and diffused back to B the process might repeat itself indefinitely, giving a periodic transfer of water from B to A.

It would be necessary to have some mechanical restraint to prevent indefinite expansion of the cell due to the incoming water. Such restraint is provided in plants by the cellulose wall and in animals by the mechanical properties of the tissue.

This mechanism may bring about the secretion of water as described in a previous paper.[8]

A thermodynamical treatment of the possible effectiveness of metabolism in causing movement of water has been given by Franck and Mayer.[9] It would seem from this that a rather high degree of efficiency is possible.

[8] Osterhout, W. J. V., *J. Gen. Physiol.*, 1947, **30**, 439.

[9] Franck, J. and Mayer, J. E., *Arch. Biochem.*, 1947, **14**, 297.

I wish to thank Mr. Jerome S. Fass for the care and skill he has shown in carrying out these experiments.

SUMMARY

The transport of water from concentrated to dilute solutions which occurs in the kidney and in a variety of living cells presents a problem of fundamental importance.

If the cell acts as an osmometer we may expect to bring about such transport by creating an inwardly directed osmotic drive which is higher in one part of the cell than in other regions of the same cell. The osmotic drive is defined as the difference between internal and external osmotic pressure.

Experiments with *Nitella* show that this expectation is justified. If water is placed at one end of the cell (A) and 0.4 M sucrose with an osmotic pressure of 11.2 atmospheres at the other end (B) water enters at A, passes along inside the cell, and escapes at B leaving behind at B the solutes which cannot pass out through the protoplasm. Hence the internal osmotic pressure becomes much higher at B than at A. When 0.4 M sucrose at B is replaced by 0.3 M sucrose with an osmotic pressure of 8.1 atmospheres we find that water enters at B, passes along inside the cell, and escapes at A so that water is transported from a concentrated to a dilute solution although the difference in osmotic pressure of the 2 solutions is more than 8 atmospheres. The solution at B thus becomes more concentrated.

It is evident that if metabolism produces a higher osmotic pressure and consequently a higher inwardly directed osmotic drive in one region of the cell as compared with other parts of the same cell water may be transferred from a concentrated to a dilute solution so that the former solution becomes still more concentrated.

[Reprinted from THE JOURNAL OF GENERAL PHYSIOLOGY, July 20, 1949,
Vol. 32, No. 6, pp. 705-724]

CRYSTALLINE PNEUMOCOCCUS ANTIBODY

BY JOHN H. NORTHROP AND WALTHER F. GOEBEL

(From the Laboratories of The Rockefeller Institute for Medical Research, New York City, and Princeton, New Jersey)

(Received for publication, February 24, 1949)

The preparation of crystalline diphtheria antitoxin was reported in this *Journal* in 1942 (Northrop, 1942). The method of purification consisted in digesting the toxin-antitoxin precipitate with trypsin, which destroyed the toxin and liberated the antibody. The antibody itself was apparently also split during the reaction so that the product obtained was a derivative of the naturally occurring antibody. The present work was undertaken to see whether this method of purification was of general application. Type I antipneumococcus horse serum was used as the starting material and the writers are indebted to Dr. Jules Freund of the New York Department of Health for supplying them with large quantities of this serum.

In preliminary experiments the antibody was precipitated by the addition of Type I specific polysaccharide and the resulting precipitate treated with trypsin or pepsin at various hydrogen ion concentrations. The antibody complex in this case, however, is not hydrolyzed, as in the case of diphtheria antitoxin and no antibody could be recovered by this process. It appears, therefore, that the action of pepsin and trypsin on antigen-antibody complexes is determined by the chemical nature of the antigen in spite of the fact that hydrolysis occurs somewhere in the antibody molecule. This result is indicative of a primary valence union between antigen and antibody, as otherwise the chemical nature of the antigen would hardly be expected to influence the hydrolysis of the antibody.

Various other methods have been described for preparing pure (completely precipitable) antibody. Landsteiner, Gay and Chickering, Felton, Kirk and Sumner, Heidelberger and Kendall, and others (*cf.* Landsteiner, 1945) obtained preparations which were pure or nearly pure antibody, in the sense that they were completely precipitated by antigen, by dissociating the antigen-antibody complex in various ways. Chow and Goebel (1935) obtained similar preparations by precipitation of a concentrated antibody solution with acid potassium phthalate solution.

Preliminary experiments indicated that the latter method was the most efficient for the preparation of large amounts of pure antibody and a slight modification of this method was used in the present work. The method of preparation finally worked out consists essentially of the following steps. High titer antipneumococcus horse serum is diluted with water and the precipi-

tate, which contains nearly all the antibody (Felton, 1928), dissolved in normal saline. This solution is mixed with 0.2 M pH 3.6 acid potassium phthalate in certain proportions, which vary slightly with different lots of serum. This treatment precipitates the inert globulins and leaves the antibody in solution. The antibody obtained in this way is 90 to 100 per cent precipitable with the specific polysaccharide. It gives a slightly milky solution in neutral salts and is nearly homogeneous in the Tiselius apparatus with a migration velocity of 1.2×10^{-5} cm.^{2}/volt sec. which corresponds to that of globulin from normal horse serum. (The writers are indebted to Dr. Gertrude Perlmann for carrying out these determinations.)

This antibody solution may be further fractionated by precipitation with ammonium sulfate into three main fractions, one of which is insoluble in neutral salts from pH 4 to pH 10, one which is soluble in neutral salts but is precipitated by 0.20 saturated ammonium sulfate. The remaining fraction precipitates between 0.20 and 0.35 saturated ammonium sulfate. It gives a clear bluish solution in neutral salts and contains most of the inert protein which survives the treatment with acid potassium phthalate.

The fraction precipitating at 0.2 saturated ammonium sulfate can be further separated into a fraction precipitating at 0.17 saturated ammonium sulfate and a small amount of antibody precipitating between 0.17 and 0.20 saturated ammonium sulfate. This last fraction may be crystallized by slowly stirring a saturated solution at 25° (in about 0.2 saturated ammonium sulfate) (Fig. 1). The crystals appear as rosettes, sometimes mixed with rods (Fig. 2). The faces are somewhat rounded in most cases and no really satisfactory preparation was obtained. Similar results have been observed in this laboratory with other proteins and have been due in most cases to the presence of more than one protein, or to the fact that conditions used for crystallizations are not quite correct. A large number of variations in conditions and precipitating agents were tried with no improvement and it is probable that the difficulty arises from the fact that the protein is unstable under the conditions required for crystallization and is partly changed to a less soluble form. This is indicated by the fact that the first crystals to appear are usually the best. Also, the insoluble protein formed during the first crystallization must be removed before recrystallization, otherwise the second crystallization yields much poorer crystals than the first. If the solution is not stirred, crystallization may start but soon stops owing to the fact that the crystals settle rapidly to the bottom of the beaker. The crystallization of pepsin is very similar since in this case also crystallization is much better and faster if the solution is stirred.

The largest part of the antibody finally collects in the fraction insoluble in neutral salts but soluble in dilute acid and alkali. The results indicate that some of this fraction is present in the original material while more is formed during the fractionation. If alkaline solutions of this insoluble protein are

mixed with solutions of the more soluble fraction, and then neutralized, the protein does not precipitate but the solution becomes cloudy. The original material used in these experiments had been stored at 5°C. for from 4 to 7 years in the presence of 0.5 per cent ether, 0.3 per cent phenol, and 0.005 per cent merthiolate. A precipitate had formed and settled out during this time and this precipitate resembles closely the insoluble protein isolated during the fractionation.

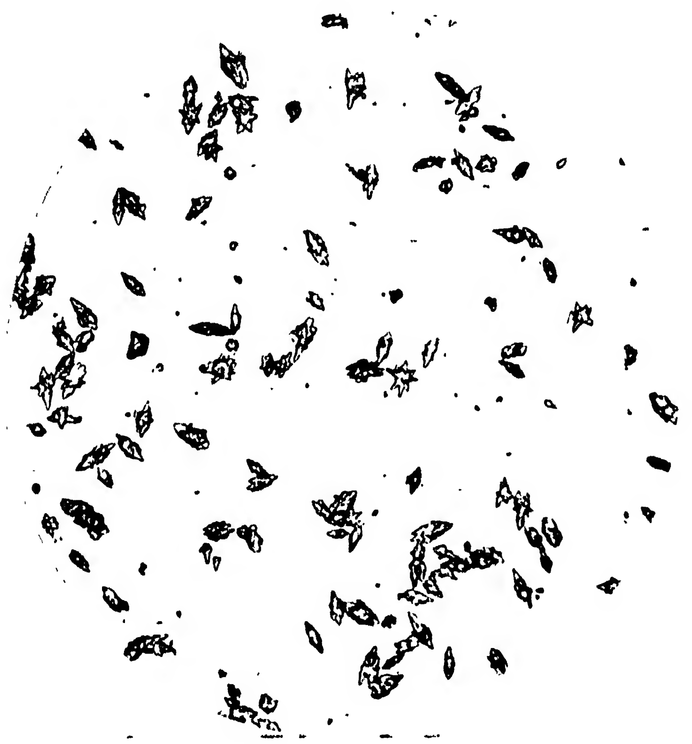


FIG. 1. Crystals of Type I pneumococcus antibody, preparation 152-51. × 130.

The most soluble fraction gives a clear, bluish solution in neutral salts and yields highly refractile rounded particles but no definite crystals. This fraction is usually 80 to 90 per cent precipitable and no method was found to remove the inert protein.

None of these fractions is even approximately homogeneous by the solubility test (Fig. 3) and crystallization does not improve the purity as determined either by solubility or by precipitation with antigen. In fact, the crystalline material is, if anything, less pure since the protein is somewhat unstable under the conditions required for crystallization so that the formation of the crystals

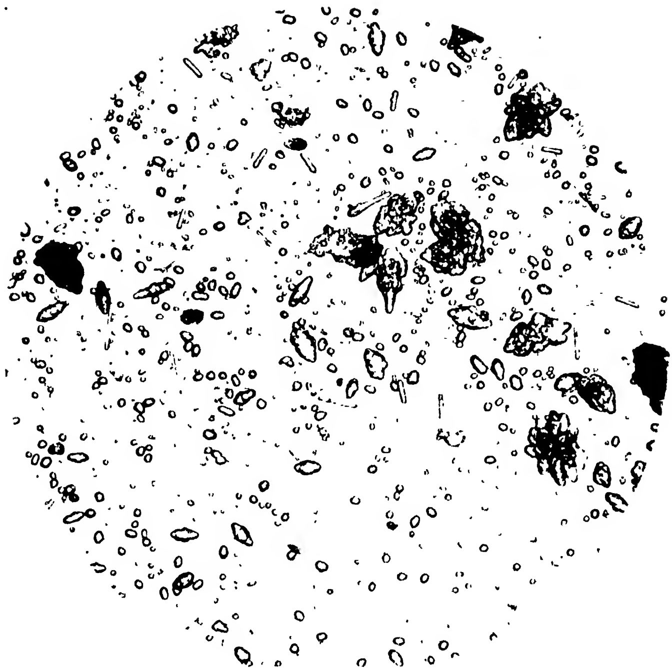


FIG. 2. Rosettes and rods. No. 72 201. × 65.

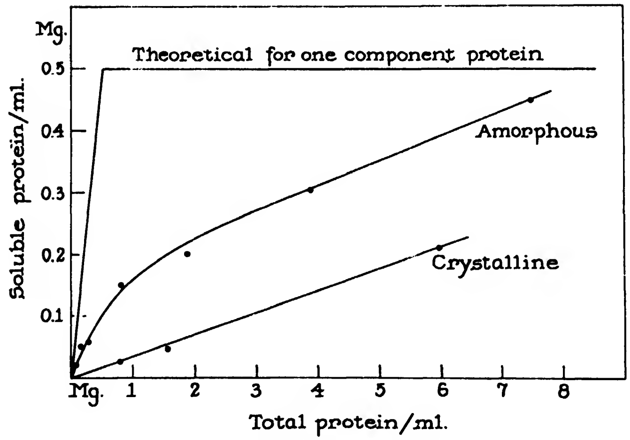


FIG. 3. Solubility of preparation 180-69 in amorphous and crystalline form in 0.33 saturated ammonium sulfate 0.17 M sodium acetate at 25°C. Two 5 ml. samples of suspension 180-69 were centrifuged. The precipitate in one tube was dissolved in 5 ml. 0.25 M sodium acetate (milky solution) and precipitated by the addition of 3 ml. saturated ammonium sulfate. This is the amorphous sample. Both crystalline and amorphous precipitates were washed four times with 2 ml. of the solvent and solubility determinations made on varying quantities of the precipitate as described in Crystalline Enzymes (Northrop, Kunitz, and Herriott, 1948).

is accompanied by the formation of some less soluble protein. The same phenomenon was encountered in the crystallization of diphtheria antitoxin (Northrop, 1942).

A purified antibody preparation was also obtained by dissociating the immune precipitate. Immunological tests of these preparations showed that they differed quite markedly in their protective and agglutinating properties. They all reacted with rabbit antihorse serum.

EXPERIMENTAL

Methods

1. Protein Determination[1].—One ml. of solution containing 0.01 to 0.5 mg. protein per ml. is added to 9 ml. 0.3 M trichloroacetic acid. The tubes are placed in a boiling water bath for 1 minute and allowed to stand 1 hour or more at 25°. The turbidity of the samples is determined in a Duboscq type Klett photoelectric colorimeter, using 0.04 M $CuSO_4$ in 0.04 M H_2SO_4 as standard. The amount of protein is determined by comparing the observed reading with a standard curve prepared from determinations made on a series of samples of known protein concentration. The suspensions do not obey Beer's law and hence the values for the concentration cannot be calculated from the colorimeter reading in the usual way but must be read off from an empirical curve as described above. The slope of the curve varies with different proteins so that it is necessary to carry out the determinations with two different concentrations of protein.

If the two determinations agree, the result is probably correct within 5 per cent. If they do not agree, the protein is different from that used to prepare the standard curve and a new standard curve must be prepared. In the present experiments, the important figure is that for protein not precipitated by the polysaccharide. In most cases this is less than 10 per cent of the total protein, so that a 10 per cent error in the determination of non-precipitable protein represents an error of 1 per cent or less in the values for the precipitable protein (antibody-protein). If the precipitable protein is less than half the total protein, however, the error is much larger and the method cannot be used for protein solutions containing low proportions of antibody.

2. Determination of Antibody Content.—The solution is diluted with 0.1 M pH 7.6 phosphate buffer to contain 0.3 to 0.8 mg. protein per ml. Two ml. of the diluted solution is placed in each of four small test tubes and 1 drop of specific polysaccharide solution containing 0.5, 1.0, or 2.0 mg. polysaccharide per ml. added to each of three tubes. All four tubes are placed at 5°C. for 24 hours, centrifuged, and the protein determined in the supernatant. The lowest value found in the tubes containing polysaccharide is taken as the figure for the inert protein and the difference between this and the protein content of the control tube is the antibody-protein. Ammonium sulfate interferes with this determination if present in concentrations greater than 0.01 saturated.

3. Precipitation with Acid Potassium Phthalate.—The results obtained with this method depend on the concentration of protein, the pH and concentration of the acid

[1] The analytical work reported in this paper, and part of the experimental procedure, were carried out by Miss Marie King.

potassium phthalate, the temperature, and the length of time the solution is allowed to stand (*cf.* Chow and Goebel, 1935). Preliminary experiments indicated that the highest yields of completely precipitable antibody were obtained by adding 0.2 M pH 3.6 acid potassium phthalate to globulin solutions containing 25 to 30 mg. protein per ml. and allowing the solutions to stand 24 hours at 20°C. The exact amount of phthalate required varies with different lots of serum. In some cases the yield of antibody is the same over quite a wide range of potassium acid phthalate, but in some cases the correct range is quite narrow. In order to determine the optimum quantity of acid potassium phthalate to add to the antibody solution, 10 ml. of solution containing 25 to 30 mg. protein per ml. is pipetted into a series of tubes and varying quantities of pH 3.6, 0.2 M acid potassium phthalate added slowly with constant stirring. A precipitate forms at once and increases slowly for several hours. After 24 hours at 20°C. the suspensions are centrifuged and the supernatants analyzed. The results of two such preliminary tests are shown in Table I. Sample 180-6 gave

TABLE I

Purification of Crude Antibody Solutions by the Addition of Acid Potassium Phthalate

Amount of 0.2 M pH 3.6 acid potassium phthalate added to 10 ml. of solution	Per cent of total protein precipitated by polysaccharide		Antibody recovered	
	Sample 180-6	Sample 170-3	Sample 180-6	Sample 170-3
ml.			*mg.*	*mg.*
0	60	60	200	200
3	90	98	200	200
3.5		98		200
4	96	100	200	140
5	98		180	
6	100		170	
7	100		120	

pure antibody with the addition of 6 or 7 ml. phthalate whereas Sample 170-3 gave pure antibody with 4.0 ml.

The mechanism of the reaction is obscure. It does not appear to be either a salting out or a denaturation. The quantity of phthalate required is nearly proportional to the amount of serum. The precipitate formed does not digest with trypsin as rapidly as does a boiled suspension of the same protein. The protein, therefore, is probably not denatured. It seems probable that the precipitate is an insoluble phthalate salt of the inert globulins. In cases where the first precipitation fails to remove all the inert protein, no further purification is obtained by repeating the phthalate treatment.

Conditions for Crystallization

The amorphous protein precipitate is stirred slowly with about three times its volume of 0.05 saturated ammonium sulfate at 5°C. for 24 hours. The protein should dissolve completely to form a clear, bluish solution. If there is more than a slight turbidity, the suspension should be filtered by gravity through No. 3 Whatman

paper, or centrifuged at high speed. The protein is then precipitated in the amorphous form by bringing the solution to 0.25 saturated ammonium sulfate, the precipitate centrifuged out and dissolved in 0.05 saturated ammonium sulfate, as described above.

If the precipitate forms a clear or nearly clear solution, the protein content is determined and the solution diluted to give a protein concentration of about 15 mg./ml. The pH is adjusted to about 6.5 and the solution stirred slowly. The stirring rod should be near the bottom of the beaker and should not cause any foam to form. Saturated ammonium sulfate is added very slowly from a dropping funnel until the

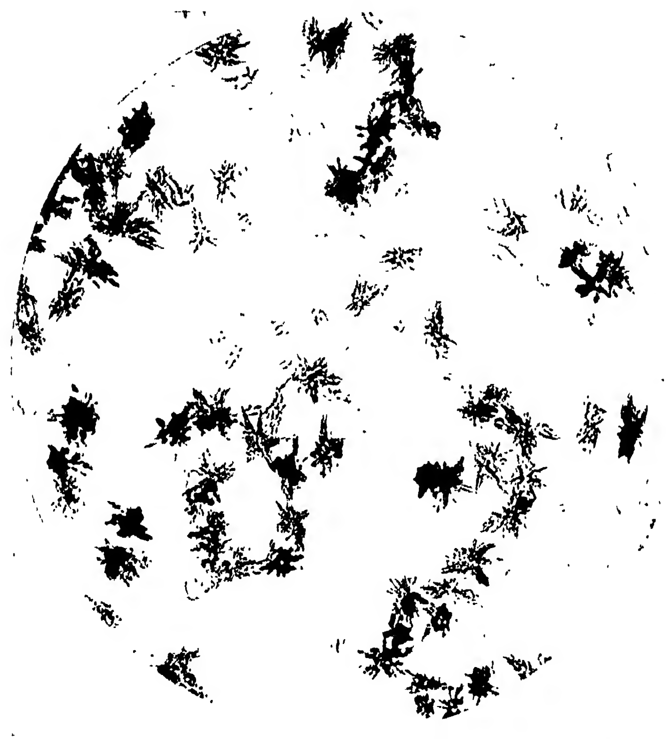


FIG. 4. Antibody "flakes," preparation 171-13, June 1, 1948.

solution is slightly cloudy. The stirring is continued and the precipitate should become rapidly heavier.

The precipitate is examined under the microscope and should consist of small irregular rosettes. The points look sharp under a low power (50 to 70 diameters) but under higher magnification appear rounded. Some preparations yield large, regular flakes, which look very much like snowflakes (Fig. 4). They appear to be made up of very small refractile granules arranged in a more or less regular pattern. These "flakes" are insoluble in 0.05 saturated ammonium sulfate and should be filtered off, if they form. If the precipitate is amorphous, it should be filtered off and the filtrate stirred at about 25°C., without the addition of more saturated ammonium

Diagram 1

Preparation of Crystalline Fractions 152-51 and 152-521

2 L Type I pneumococcus antibody solution
diluted to 12 L with dist water, 5°, 72 hrs,
decant and filter ppt -susp + hyflo

↓

Ppt extracted with 2L 0 2M NaCl twice
Extract 6 dil +1.3 L 0 2M NaCl, 7, (60%),
5 3 L 7 + 2 6 L 0 2 M pH 3 6 KH-phthalate, 18°, 20 hrs,
decant, filter ppt-susp + S S C *
supernatant + filtrate 10, 90%,
6 8 L 10 + 1.7 L SAS (0 2 sat), 25°, 20 hrs,
siphon supernatant and centrifuge ppt-susp

↙ ↘

Ppt 12
+ 600 ml 0 2M pH 7 6 PO_4, filter + S S C
↓
Ft. 21
Add SAS to 0 2 sat, 24 hrs, 25°, filter
↓
Ppt 23P

Sup 11
Add SAS to 0 3 sat, 25°, 20 hrs, decant and filter
↓
Ppt. 13
+2 L 0 2M pH 7 6 PO_4 + SAS to 0 2 sat. 25°, 24 hrs,
decant and filter
↓
Ppt 15P

↘ ↙

23P + 15P + 1 L 0 05 SAS, filter
↓
Ft 24
Add SAS to 0 25 sat, 25°, 20 hrs, filter + S S C.
↓
Ppt 26P
Extract with 2L 0 1 M pH 7 6 PO_4, extract 29, 100%, add SAS to 0 17 sat,
flaky ppt, filter, cloudy filtrate, add S S C and filter
↓
Ft.
Stir and add SAS till sl cloudy, 5°, 24 hrs, flaky ppt, centrifuge

↙ ↘

Ppt 41
Dissolve in 50 ml H_2O, nearly clear, filter #3, grav, filtrate + 100 ml H_2O, stir & add SAS till cloudy, stir 25°, amorph ppt, filter #3, clear, stir & add SAS till sl cloudy, granular ppt, suspension 5°, 1 mo. — cent.
↓
Ppt + 50 ml H_2O, clear, stir & add SAS till sl cloudy, stir 25°, 2 days, good rosettes, cent
↓
Ppt + 10 ml H_2O, nearly clear, add SAS till sl cloudy, filter, add 10 ml. H_2O, stir & add SAS till sl cloudy, stir 25°, 2 days, good rosettes — 51, 97%

Sup 42
Stir & add SAS till sl cloudy, stir 24 hrs, 25°, gran. ppt., cent.
↓
Ppt + 100 ml. H_2O, nearly clear, stir & add SAS till sl. cloudy, rods & rosettes, 1 mo, 5°, 52, dilute with equal vol. H_2O, stir 25°, poor rosettes, filter, ppt dissolved in 40 ml H_2O, stir & add SAS till sl cloudy, filter
↓
Filtrate, clear, bluish, stir & inoculate from 51 24 hrs., poor rosettes — 521, 95%, cent.
↓
Ppt. + 30 ml H_2O, clear, add SAS till sl. cloudy, stir 24 hrs., 25°, large flakes & poor rosettes, cent
↓
Ppt + 30 ml. H_2O, 5°, 24 hrs, stir & add SAS till sl. cloudy, stir 25°, 24 hrs, heavy ppt., good rosettes, 521

* S S C. indicates Standard Super Cel.

TABLE II

Experiment 152

	No.	Vol.	Protein/ml.	Antibody	Total AB
		ml.	*mg.*	*per cent*	*gm.*
6 liters of Type I antipneumococcus horse serum diluted to 60 liters with distilled water, 5°C., 48 hrs. Supernatant siphoned off and precipitate centrifuged. Precipitate dissolved in 2 liters 0.1 M NaCl. 0.5 per cent ether, 0.3 per cent phenol, and 0.005 per cent merthiolate added and the solution allowed to stand at 5°C. for 6 yrs.	1	2000			
2 liters *No. 1* diluted to 12 liters with water. allowed to stand 72 hrs. at 5°C., supernatant decanted, precipitate-suspension (4 liters) + 200 gm. Hyflo, filtered by suction on 235 cm. E and D No. 303 papers, precipitate + 2 liters 0.2 M NaCl, filtered	4	2000			
Precipitate + 2 liters 0.2 M NaCl, filtered	5	2000			
No. 4 + 5	6	4000	36	60	80
4 liters *No. 6* + 1.3 liters 0.2 M NaCl	7	5300	27	60	80
5.3 liters *No. 7* + 2.6 liters 0.2 M pH 3.6 potassium acid phthalate, 18°C., 20 hrs., supernatant siphoned off, supernatant + 200 gm. standard Super Cel, filtered on 35 cm. No. 303 E and D paper... Filtrate	8				
Precipitate-suspension + 100 gm. Hyflo + 100 gm. standard Super Cel, filtered.........Filtrate	9				
No. 8 + 9	10	6800	9	90	55
6.8 liters *No. 10* + 1.7 liters saturated ammonium sulfate (0.2 saturated) 25°C., 20 hrs., supernatant siphoned off.........Supernatant	11				
Precipitate-suspension	12				
6 liters *No. 11* + 2 liters saturated ammonium sulfate (0.3 saturated), 25°C., 20 hrs., supernatant siphoned off, precipitate-suspension filtered on No. 3 paper...					
Precipitate	13				
No. 13 + 2 liters 0.2 M pH 7.6 phosphate buffer	14	2200	20	90	40
2.2 liters *No. 14* + 550 ml. saturated ammonium sulfate (0.2 saturated) 25°C., 24 hrs., filtered on folded No. 3 paper.........Precipitate	15P				
Precipitate-suspension *No. 12* centrifuged, precipitate + 600 ml. 0.2 M pH 7.6 phosphate buffer + 40 gm. standard Super Cel, filtered.........Filtrate	21	600	30		
Precipitate	21P				
600 ml. *No. 21* + 150 ml. saturated ammonium sulfate (0.2 saturated) 24 hrs., 25°C., filtered on folded No. 3 paper.........Precipitate	23P				

TABLE II—*Continued*

	No.	Vol.	Protein/ml.	Antibody	Total AB
		ml.	*mg.*	*per cent*	*gm.*
No. 23P + 15P stirred with 1 liter 0.05 saturated ammonium sulfate, filtered by suction on 25 cm. No. 303 E and D paper................Filtrate......	24	1000			
1 liter *No.* 24 + 250 ml. saturated ammonium sulfate (0.25 saturated), stirred slowly at 25°C. 24 hrs. + 30 gm. standard Super Cel, filtered...Precipitate...	26P				
No. 26P + 1500 ml. 0.1 M pH 7.6 phosphate buffer, stirred, filtered, milky..............Filtrate......	27				
Precipitate + 500 ml. 0.1 M pH 7.6 phosphate buffer, stirred, filtered.................. ..Filtrate......	28				
No. 27 + 28.......	29	2000	14	100	28
800 ml. *No. 29** + 160 ml. saturated ammonium sulfate (0.17 saturated), stirred in slowly, flaky precipitate, filtered, slightly cloudy.............Filtrate......	30	1000	9	95	9
Filtrate *No. 30* + 10 gm. standard Super Cel, filtered by suction on 20 cm. No. 3 paper, bluish filtrate, stirred, and saturated ammonium sulfate added slowly until slightly cloudy, 5°C., 24 hrs., granular precipitate, decanted and centrifuged. ....Precipitate...	41				
Supernatant..	42				
Precipitate *No. 41* + 50 ml. water, nearly clear, filtered on No. 3 paper, stirred and saturated ammonium sulfate added until slightly cloudy, amorphous precipitate. 100 ml. water added, clear solution, saturated ammonium sulfate stirred in until slightly cloudy, filtered on No. 3 paper. Saturated ammonium sulfate added until slightly cloudy and stirred, granular precipitate, allowed to stand at 5°C. for 1 mo. Centrifuged, precipitate + 50 ml. water, clear, stirred, and saturated ammonium sulfate added until very slightly cloudy and stirred at 25°C. 2 days, good rosettes. Centrifuged, precipitate + 10 ml. water, nearly clear, saturated ammonium sulfate added until slightly cloudy, filtered, 10 ml. water added, stirred and saturated ammonium sulfate added until slightly cloudy, stirred at 25°C. 2 days, good rosettes	51	60	56	97	3
No. 42 stirred and saturated ammonium sulfate added slowly until slightly cloudy, stirred 24 hrs., granular precipitate, centrifuged, precipitate + 100 ml. water, nearly clear, stirred and saturated ammonium sulfate added until slightly cloudy, rods and rosettes, allowed to stand 1 mo. at 5°C..................	52				

TABLE II—*Concluded*

	No.	Vol.	Protein/ml.	Antibody	Total AB
		ml.	*mg.*	*per cent*	*gm.*
Diluted with equal volume of water, stirred 24 hrs., poor rosettes, filtered, precipitate dissolved in 40 ml. water, stirred and saturated ammonium sulfate added until slightly cloudy, filtered by gravity on No. 3 paper, bluish filtrate, stirred and inoculated with *No. 51*, stirred 24 hrs., poor rosettes..........	521	50	18	95	.9
Centrifuged, precipitate + 30 ml. water, stirred and saturated ammonium sulfate added until slightly cloudy, stirred 24 hrs. at 25°C., large flakes and poor rosettes, centrifuged, precipitate + 30 ml. water, 5°C., 24 hrs., stirred and saturated ammonium sulfate added until slightly cloudy, 25°C., 24 hrs., heavy precipitate, good rosettes					

* The remainder of solution *29* was used in other fractionation procedures, which failed to yield crystalline material.

sulfate. A crystalline precipitate should form in an hour or so and most of the protein should be out of solution after 24 to 36 hours. Recrystallization is carried out in the same way. The crystals dissolve quite slowly and 24 hours' stirring is required to complete the solution. There is always more or less insoluble protein left, after the crystals have dissolved.

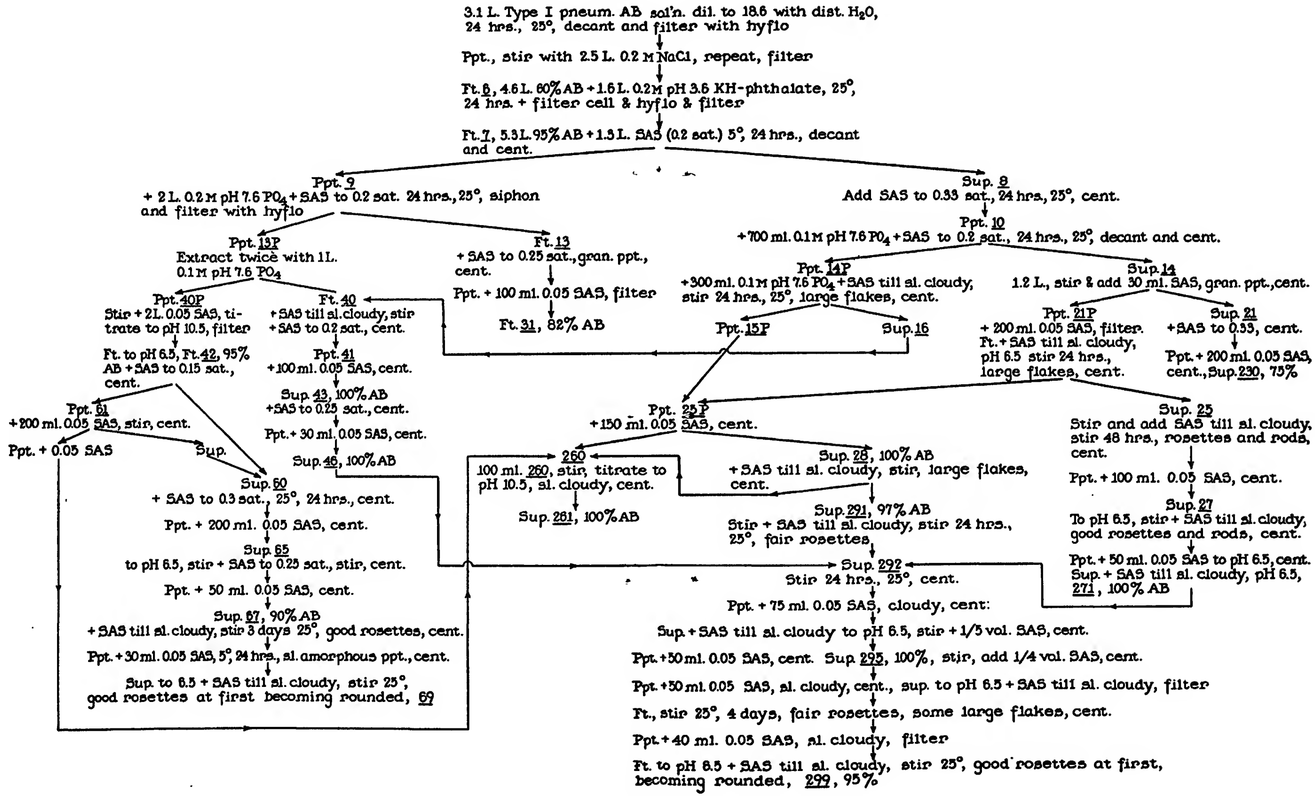
Preparation of Various Antibody Fractions

The method of preparing one sample of crystalline antibody is shown in outline in Diagram 1 and in detail in Table II. Crystalline material was obtained from four different lots of antibody solution and from three different lots of serum. The steps in the isolation varied somewhat in each case but in every case the crystalline material was obtained from the same fraction; that which precipitated in the range of from 0.17 to 0.2 saturated ammonium sulfate at pH 6 to 7. Some sera had a higher content of this fraction than did others so that no general figure for the total yield of crystalline material can be given. The antibody solution used in Experiment 152 (Table II) contained the highest proportion of the fraction and that reported in a subsequent experiment (Experiment 180, Diagram 2, not presented in table form) the lowest.

The fraction of antibody insoluble in neutral salts, and also the fraction precipitating at 0.17 saturated ammonium sulfate are mucilaginous and do not give clear solutions except in dilute acid, pH 3 to 4, or dilute alkali, pH 9.5 to 10.5. Precipitates of these fractions are extremely difficult to centrifuge or filter. If large amounts of filter aids, such as Filter Cel, are used, the filtration is fast, but most of the protein cannot be recovered from the Filter Cel. Filtration by gravity through folded No. 3 Whatman paper gives the clearest filtrate but is extremely slow. Centrifugation was carried out in an angle centrifuge at about 5,000 R.P.M.

Diagram 2

Fractionation Experiment 180



Preparation of Dissociated Antibody and Solutions of Antibody Used in Immunological Tests

The immune precipitate was prepared from the stock antibody solution used to prepare crystalline fraction 152-52. The method of preparation was the same as that described by Heidelberger and Kendall (1936) as modified by Goebel, Olitsky, and Saenz (1948). This preparation was 80 per cent precipitable by the homologous polysaccharide.

Stock solutions of the crystalline antibody were prepared as follows. Approximately 100 mg. of each of the crystalline antibody fractions, suspended in ammonium sulfate, was centrifuged at low speed, and the supernatant liquid decanted. The material was taken up in 5 ml. of 0.2 M sodium chloride and permitted to stand at room temperature for 6 hours, then overnight in the ice box. The substances were dialyzed free of $SO_4^=$ against a 0.2 M solution of sodium chloride containing 0.02 M sodium phosphate buffer at a pH of 7.2, and analyzed for their nitrogen content. Merthiolate was added so that the final concentration was 1 part in 20,000. These stock solutions were stable, and contained approximately 2 to 3 mg. of antibody nitrogen per ml. They were diluted to the appropriate concentration with sterile physiological salt solution prior to their use in the experiments described below.

Fraction No. 180–260 was brought into solution in the following manner. Approximately 2 ml. of the suspension of the immune protein was stirred with 50 ml. of 0.2 M phosphate buffer at pH 7.4. To this was added with stirring 2.0 ml. of M/1 sodium hydroxide. The final pH of the solution was 9.8. Part, but not all, of the protein dissolved. The material was dialyzed at 6°C. against successive changes of a 0.2 M phosphate buffer which had also been adjusted to pH 9.8, until free of ammonium sulfate. The solution was then analyzed for total nitrogen and then centrifuged after adjusting the pH to 7.8. Approximately 60 per cent of the total protein nitrogen remained in solution.

Comparison of the Immunological Properties of the Crystalline and Dissociated Antibody

1. Agglutination of Types I and II Pneumococci.—In order to determine the maximum dilution at which the crystalline antibody protein would agglutinate Type I pneumococci, serial dilutions of the solution of fraction 152-52 were prepared, and tested against a suspension of a 6 hour culture of washed Type I microorganisms killed by heating for 30 minutes at 60°C. 0.5 ml. of the appropriate antibody dilution in 0.9 per cent NaCl was mixed with 0.5 ml. of a suspension of pneumococci. The tubes were incubated at 37°C. for 2 hours, and after standing at 5°C. overnight, the agglutination reactions were recorded. For purposes of comparison a solution of the dissociated antibody was at the same time tested, as were the agglutination reactions of Type II pneumococci in solutions of both antibodies. The results recorded in Table III show that the minimum amount of crystalline antibody protein required to cause agglutination of Type I pneumococci was 8 micrograms of antibody nitrogen, whereas twice this amount of the dissociated antibody protein was necessary to cause comparable agglutination. It can also be seen from Table III that the agglutination was specific, for neither antibody agglutinated Type II pneumococci.

2. Protective Action of Type I Pneumococcal Antibody Fractions.—The protective power of the various crystalline and amorphous antibody protein fractions was tested by injecting 20 gm. Swiss mice intraperitoneally with a fixed quantity of virulent Type I pneumococci and varying quantities of the antibody proteins. 0.5 ml. of each antibody dilution, together with 0.5 ml. of a 6 hour culture of Type I pneumo-

TABLE III

Agglutination Reactions of Type I and II Pneumococci in Type I Crystalline Pneumococcal Antibody and in Dissociated Type I Antibody

Antibody fraction tested	Pneumococci type	Antibody nitrogen in sample tested, µg.					
		64	32	16	8	4	2
Fraction 152-52	I	++++	++++	++++	++	0	0
Dissociated antibody	I	++++	+++	++	0	0	0
Fraction 152-52	II	0	0	0	0	0	0
Dissociated antibody	II	0	0	0	0	0	0

TABLE IV

Protective Action of Pneumococcal Antibody Protein Fractions against 10^6 L.D. Type I Pneumococci

Antibody fraction tested	Antibody nitrogen injected, µg.									Antibody N protecting 50 per cent of mice
	40	20	10	5	2.5	1.25	0.62	0.31	0.16	µg.
180- 69			5/6	6/6	2/6	0/6	1/6			2.9
180-299			6/6	6/6	4/6	1/6	0/6			1.9
152- 52			6/6	5/6	3/6	0/6	0/6			2.8
180-260	5/6	5/6	6/6	2/6	2/6	0/6.				6.3
Dissociated			6/6	6/6	6/6	6/6	4/6	0/6	0/6	0.5

Virulence controls: 10^{-6} 0/3
10^{-7} 0/3
10^{-8} 2/3

Plate count: 10^{-7} 12, 13 colonies
10^{-8} 4, 1 colonies

Numerator, survivals. Denominator, deaths.

cocci containing 2×10^6 microorganisms per ml., was injected into each of six mice. The virulence of the bacterial culture was such that 0.5 ml. of a 10^{-8} dilution, containing one to two microorganisms, killed mice when injected intraperitoneally. The animals were observed, after injection, for a period of 5 days and their deaths recorded in Table IV.

From the results presented in Table IV it can be seen that the amount of crystalline antibody protein nitrogen necessary to protect half the animals

against a million lethal doses of Type I pneumococci was approximately 2 to 3 micrograms. On the other hand, it required 6.35 micrograms of antibody nitrogen of the amorphous fraction No. 180-260[2] to afford comparable protection, whereas only 0.5 microgram of the dissociated antibody was necessary to give equal protection. It can also be seen in Table V that the protective action of the crystalline antibody is type-specific, for the protein affords no protection to mice infected with virulent Type II pneumococci.

In summarizing the results of the protection experiments it may be stated that the crystalline antibody fractions all had approximately the same protective action, based on the antibody nitrogen content, whereas the amorphous fraction No. 180-260 which formed by far the greater part of the antibody obtained during the process of crystallization was low in protective value. The dissociated antibody, on the other hand, was more potent than any of the fractions tested.

TABLE V

Protective Action of Crystalline Pneumococcal Antibody Fraction 152-52 against 10^6 L.D. Type II Pneumococci

Antibody fraction tested	Antibody injected, µg.			
	32	16	8	4
152-52	0/3	0/3	0/3	0/3

Virulence controls: 10^{-7} 0/3 Plate count: 10^{-7} 28, 31
10^{-8} 0/3 10^{-8} 4, 2

3. *Precipitin Reactions of Pneumococcal Antibody Fractions.*—In order to determine whether the various pneumococcal antibody fractions were immunologically related to the globulins present in normal horse serum, the fractions were tested in antiserum prepared by the immunization of rabbits with normal horse serum. Such an immune serum will contain antibodies directed against all the proteins present in normal horse serum. If pneumococcal antibody is a protein dissimilar to the proteins in normal horse serum, there should be no precipitin reaction when the test is performed. Preliminary experiments showed that all the fractions reacted in an antiserum to normal horse serum.

In order to determine whether differences in the intensity of the reactions could be observed, the precipitin tests were carried out quantitatively using a photo-turbidimeter (Libby, 1938). Results of these experiments are represented graphically in Fig. 5.

[2] This fraction when first prepared was completely precipitable with the specific polysaccharide. At the time the above experiments were performed it was only 72 per cent precipitable.

From the results presented it can be seen that the crystalline fractions 152-52, 180-69, and 180-299 reacted all with nearly equal intensity, whereas fraction 180-260, the amorphous material, gave a reaction which was somewhat stronger. The amorphous dissociated immune globulin, on the other hand, gave a reaction the intensity of which was considerably lower than that of the crystalline proteins.

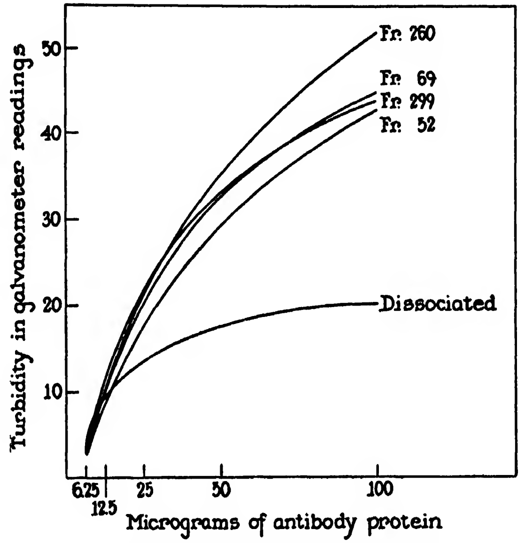


FIG. 5. Turbidimetric titration of pneumococcal antibody proteins in an antiserum to normal horse serum.

DISCUSSION

The present experiments show that it is possible to prepare a series of protein fractions from antipneumococcus horse serum, all of which are completely or nearly completely precipitated by the specific polysaccharide but which differ in their solubility, protective value, agglutinating power, and reaction with antihorse rabbit serum (Table VI). One of the fractions may be obtained in poorly crystalline form.

These results show quite conclusively that a whole series of proteins exists which precipitate specifically with the polysaccharide. It appears extremely unlikely, for instance, that a protein fraction which is completely insoluble in dilute neutral salts (fraction 180–260) could contain enough of a soluble protein, such as fraction 152-52, to cause the whole complex to precipitate completely with the polysaccharide. Whether the proteins exist as such in the serum or are modified during the fractionation is uncertain. The results also indicate that these immune proteins possess both protective and precipitating proper-

ties in varying proportion, as suggested by Heidelberger and Kendall (1935) and Goodner and Horsfall (1937).

The crystalline diphtheria antitoxin (Northrop, 1942) also protected against, as well as precipitated with, the toxin. This preparation was very nearly homogeneous and hence there is reason to think that in this case, at least, the same protein may possess both precipitating and protective properties.

This preparation did not precipitate with rabbit antihorse serum. This may be due to the fact that it was much more homogeneous than the present

TABLE VI

Summary of Properties of Various Antibody Preparations

Fraction	Solubility		Per cent precipitable + polysaccharide	Amount required to protect	Amount required to agglutinate	Reaction with rabbit antihorse serum
	Soluble in	Insoluble in				
				μg.	μg.	
Dissociate antibody	0.1 M NaCl	H_2O	80	0.5	16	+
152-52 Crystalline	0.1 M NaCl 0.15 saturated ammonium sulfate	H_2O 0.25 saturated ammonium sulfate; H_2O	95	2.8	8	++
180-69 Crystalline	" "	" "	90	2.9		++
180-299 Crystalline	" "	" "	95	1.9		++
180-260 Amorphous	pH 3-5 9-10	pH 5-9 All neutral salts	100* 70‡	6.3		+++

* When first prepared.

‡ When protective test was made.

preparations or because it was changed during the digestion of the immune precipitate with trypsin.

Unfortunately, none of the present fractions is even approximately homogeneous, as judged by the solubility test (Northrop, Kunitz, and Herriott, 1948), so that it is possible that the varying protective values are due to the presence of varying quantities of a special highly active protective antibody. A definite decision between the two possibilities probably cannot be made until a strictly homogeneous immune protein is prepared.

SUMMARY

1. The immune precipitate formed by antipneumococcus horse serum and the specific polysaccharide is not hydrolyzed by trypsin as is the diphtheria

toxin-antitoxin complex, and purified pneumococcus antibody cannot be isolated by the method used for the isolation and crystallization of diphtheria antitoxin.

2. Type I pneumococcus antibody, completely precipitable by Type I polysaccharide, may be obtained from immune horse serum globulin by precipitation of the inert proteins with acid potassium phthalate.

3. The antibody obtained in this way may be fractionated by precipitation with ammonium sulfate into three main parts. One is insoluble in neutral salts but soluble from pH 4.5 to 3.0 and from pII 9.5 to 10.5. This is the largest fraction. A second fraction is soluble in 0.05 to 0.2 saturated ammonium sulfate and the third fraction is soluble in 0.2 saturated ammonium sulfate and precipitated by 0.35 saturated ammonium sulfate. The second fraction can be further separated by precipitation with 0.17 saturated ammonium sulfate to yield a small amount of protein which is soluble in 0.17 saturated ammonium sulfate but insoluble in 0.25 saturated ammonium sulfate. This fraction crystallizes in poorly formed, rounded rosettes.

4. The crystallization does not improve the purity of the antibody and is accompanied by the formation of an insoluble protein as in the case of diphtheria antitoxin.

5. None of the fractions obtained is even approximately homogeneous as determined by solubility measurements.

6. Purified antibody has also been obtained by dissociating the antigen-antibody complex.

7. The protective value of the fractions is quite different; that of the dissociated antibody being the highest and that of the insoluble fraction, the lowest.

8. All the fractions are immunologically specific since they do not precipitate with Type II polysaccharide nor protect against Type II pneumococci.

9. All the fractions give a positive precipitin reaction with antihorse rabbit serum. The dissociated antibody gives the least reaction.

10. Comparison of the various fractions, either by their solubility in salt solution or through immunological reactions, indicates that there are a large number of proteins present in immune horse serum, all of which precipitate with the specific polysaccharide but which have very different protective values, different reactions with antihorse rabbit serum, and different solubility in salt solutions.

REFERENCES

Chow, B., and Goebel, W. F., 1935, *J. Exp. Med.*, **62**, 179.

Felton, L. D., 1928, *J. Infect. Dis.*, **43**, 543.

Goebel, W. F., Olitsky, P. K., and Saenz, A. C., 1948, *J. Exp. Med.*, **87**, 445.

Goodner, K., and Horsfall, F. L., 1937, *J. Exp. Med.*, **66**, 413, 425, 437.

Heidelberger, M., and Kendall, F. E., 1935, *J. Exp. Med.*, **62**, 697.

Heidelberger, M., and Kendall, F. E., 1936, *J. Exp. Med.*, **64**, 161.

Landsteiner, K., 1945, The Specificity of Serological Reactions, Cambridge, Harvard University Press.

Libby, R. L., 1938, *J. Immunol.*, **34**, 269; **35**, 289.

Northrop, J. H., 1942, *J. Gen. Physiol.*, **25**, 465.

Northrop, J. H., Kunitz, M., and Herriott, R. M., 1948, Crystalline Enzymes, Columbia Biological Series, No. 12, New York, Columbia University Press, 2nd edition, 288.

[Reprinted from The Journal of Experimental Medicine, April 1, 1949, Vol. 89, No. 4, pp. 399–424]

ADDITIVE EFFECTS OF CERTAIN TRANSFORMING AGENTS FROM SOME VARIANTS OF PNEUMOCOCCUS

By HARRIETT E. TAYLOR, Ph.D.*

(*From the Hospital of The Rockefeller Institute for Medical Research*)

Plate 15

(Received for publication, December 9, 1948)

There has been little reason to believe that the bacterial cell contains a special genetic substance or structure, differentiated to perform genetic functions, for it can be imagined that each part of the bacterium reproduces itself in fission, and is thus itself responsible for its own genetic continuity. There are numerous examples of differentiated extranuclear structures, in nucleated cells, which are capable of a high degree of genetic autonomy, and indeed which may be said to reproduce themselves provided a favorable intracellular environment is present. For example, there are the plastids of plants and the centrosomes of animal cells. The genetic autonomy of these structures is apparently compatible with the perpetuation of the cell in constant form. In view of the fact that even in highly differentiated organisms, non-nuclear genetic elements possessing genetic continuity are found, a number of hypotheses can be proposed to explain the constant properties of bacterial clones. No definitive evidence exists as yet to indicate whether or not this constancy may be attributed to the functioning of a nuclear apparatus.

At present one type of evidence suggests that certain inherited traits in bacteria are controlled by localized units. X-rays produce permanent alterations in bacterial cells, as well as in the germ cells of higher organisms. In the latter case, the site of action of the x-rays has been localized in the nuclear genes. Although quantitative data on the production of heritable changes in bacteria suggest that here too the x-rays are acting upon certain localized structures, and not upon structures distributed generally throughout the cell, rigorous proof of the "target theory" is lacking in bacteria (1). Hence, the radiation studies are only presumptive evidence that perpetuation of the affected characters is achieved through the activities of single, localized determinants within the bacteria studied.

From an entirely different approach, evidence has been obtained that the production of at least one specific complex element of a bacterium is determined by a chemically unrelated cellular component. Thus, it appears that production of the pneumococcal type-specific capsular antigen is dependent upon the presence of a specific desoxyribonucleic acid within the bacterium. Bacteria which do not form the specific antigen may interact with isolated specific

* Work done in part as a Fellow in the Natural Sciences of the National Research Council. Present address: Institut de Génétique, 13 Rue Pierre Curie, Paris.

nucleic acid and, as a result of this interaction, their progeny continue to produce not only the specific antigen, but also more of the specific nucleic acid used to induce the change (2–4).

As the transformed organism divides, its progeny are found to form both a capsule, and the transforming principle, just as do encapsulated pneumococci of natural origin. It appears justified, therefore, to visualize the transforming principle much as the geneticist pictures genes, as a self-duplicating agent which initiates a series of reactions, involving in the case of the transforming principle probably both the synthesis of a specific enzyme and a capsular polysaccharide.

In the past, colonial morphology and immunological characteristics have served as criteria for differentiating pneumococcal cultures. As a consequence of the transformation studies, however, characterization of smooth and rough races of pneumococci may be made by yet another criterion: the gene-like transforming principle found in each of the smooth strains thus far studied, is apparently absent in the non-encapsulated or rough races (3, 4). While races of pneumococci have been analyzed with respect to their antigenic structure, no extensive study of the group has been made considering as an essential differential characteristic the presence or absence of specific transforming principles. The present study is essentially an inquiry into the differences between several races of pneumococci, as manifest in the transforming activities of desoxyribonucleates obtained from these races.

The present report will cover the following points:

1. From the R strain used customarily in transformation experiments, a permanently altered strain, ER, was obtained which forms an extremely rough colony on solid media. Evidence will be presented which indicates that strain ER differs from R in one essential feature. R pneumococci possess a transforming principle capable of transforming ER pneumococci into R, while ER pneumococci lack such a principle. SIII pneumococci possess not only the transforming principle active in transforming R pneumococci into SIII, but also the new transforming principle active in changing ER pneumococci into R.

2. Two of a series of mutated forms of Type III smooth pneumococci have been studied. The experiments to be discussed indicate that the altered condition of these strains is attributable to mutation of the transforming principle found uniquely in SIII pneumococci. Experiments upon an analogous strain of Type II pneumococcus have recently been described (5). The more extensive data to be discussed in connection with the present experiments upon the SIII variant strains provide strong evidence against the suggestion of these authors that the transformation of an R pneumococcus into an S requires the acquisition of more than one "character" by the pneumococcus undergoing transformation. Neither the material published by these authors, nor the present experiments, give information as to whether the transformation of R into S involves the action of one or more active particles of the desoxyribonu-

cleate fraction of the S cell. However, the present experiments exclude the possibility that the mutated SIII races are races in which a normally multipartite SIII transforming agent is only partially present. Phrased in the terms of genetics, the present experiments suggest that the mutated and normal SIII transforming principles are related to each other as are the genes of an allelic series, and not as genes of a polymeric series of which more than one must be present for normal antigen production.

3. Two kinds of two-step transformations were found possible. From the differences between these two-step transformations it is concluded that in certain cases the transformation phenomenon consists of an interaction or exchange occurring between a transforming principle within the pneumococcus and a similar principle in the environment.

Materials and Methods

To obtain transformation *in vitro* it is necessary to provide special environmental conditions for the susceptible pneumococcus. It has been shown that the essential components of this environment, in addition to nutrient materials, are (1) an agglutinating agent and (2) an accessory protein constituent of serum, which, when present in whole serum, can be inactivated by dialysis (6). Except for certain modifications which will be discussed below, essentially identical techniques have been employed for all the various transformations to be described, and in every case the above environmental conditions had to be met.

Agglutinating Agents

In transforming an R strain, either a whole serum was employed which contained enough R agglutinins to provide completely sedimented growth of the R inoculum, or the agglutinins were added in the form of a concentrated gamma globulin fraction of the serum of rabbits immunized with rough pneumococci. A crude fractionation of the serum was made by dialyzing the serum against 18 per cent sodium sulfate. The resultant precipitate was collected by centrifugation and washed by resuspension in 18 per cent sodium sulfate. The washed precipitate was dissolved in a small volume of distilled water and this solution dialyzed against physiological saline. Sterility was maintained throughout. Following dialysis, the agglutinating activity of the sterile solution was titered by growing R pneumococci in medium containing varying amounts of the antibody solution. This kind of titration is called a "thread test," and will be referred to by this name at several points in the following report. Most preparations of R agglutinins contained enough antibody to give completely sedimented growth of the R bacteria at a concentration of 1 part of the agglutinin solution to 1000 parts of broth. Consequently, this was the concentration usually employed for transformation experiments.

In some instances SIII organisms were transformed, and to agglutinate these pneumococci an appropriate system was substituted. Two techniques were employed. The solutions of R agglutinins could be employed to agglutinate the SIII bacteria provided the specific polysaccharide formed by these pneumococci was constantly removed from the cell surface during growth. This could be done by using the enzyme which specifically hydrolyzes the Type III polysaccharide (7) since the enzyme in no way interferes with the transformation process. As an alternative technique, Type III antiserum could be employed to agglutinate the SIII organisms, either in the form of whole rabbit serum, or in the form of a twice precipitated

gamma globulin fraction of Type III antipneumococcus horse serum. The concentrations used were sufficient to give agglutinated growth of the inoculum of SIII cells. In the case of the globulin fraction of horse serum, 1 part of the solution was added to 1000 parts of broth.

Sources of Accessory Serum Factor

The second serum constituent, which is distinct from the agglutinins, was added either as whole human pleural fluid, of known activity in supporting transformations, at concentrations of 7 to 10 per cent, or in the form of a purified fraction of bovine serum. In a future publication a report will be made on the experiments performed in collaboration with Dr. O. T. Avery and Dr. R. D. Hotchkiss, which have led to the use of bovine serum albumin in place of whole serum in effecting transformations. As routine a 4 per cent solution of fraction V of bovine serum albumin, prepared by Armour and Company according to the techniques developed in the laboratory of Dr. E. J. Cohn, was adjusted to a pH of 7.0 and filtered through Coors No. 3 porcelain filters for sterilization. This solution was added to broth in amounts ranging from 3 to 5 parts per 100, this range of concentrations having been found to be in excess of the minimum necessary to obtain regular transformations with active transforming extracts. Sodium pyrophosphate added to the transforming environment was found to eliminate some variability in the results, and consequently was added to the albumin solution just prior to its use in an experiment. 1 part of a M/15 solution of sodium pyrophosphate was mixed with 10 parts of the 4 per cent albumin solution.

Preparation of Transforming Principles

The improved method of isolating transforming principles from pneumococci was used (4). When only a few tests of biological activity of a given preparation were to be performed, large scale preparations were not made, and hence extensive purifications could not be carried out. The preparations of transforming principles made on a small scale contained several milligrams of desoxyribonucleate, and in addition contained ribonucleic acid, somatic polysaccharide, and in some cases, capsular polysaccharide. All transformations employing the normal SIII transforming principle, and all employing the desoxyribonucleate of R cells, were performed with highly purified preparations of desoxyribonucleic acid. The majority of transformations with the transforming principle referred to as SIII-1 were also done with highly purified desoxyribonucleate. The remaining preparations were made as described below.

The strain from which a transforming extract was to be prepared was grown from a single colony isolation, and a heavy inoculum seeded into 1500 cc. of beef heart infusion–neopeptone broth. After 17 hours' incubation at 37°C. the cells were harvested by centrifugation. Following resuspension in 10 cc. of 0.1 M sodium citrate–0.1 M saline solution, 0.05 cc. of a 10 per cent solution of sodium desoxycholate was added to induce lysis. Lysis usually occurred within 15 minutes at room temperature. The highly viscous lysate was then treated 3 times by the Sevag deproteinization method, employing chloroform and amyl alcohol. The still turbid lysate was then treated with 5 volumes of alcohol, in which a fibrous precipitate formed. The precipitate was allowed to remain overnight in the alcohol to assist the process of protein denaturation and to inactivate traces of pneumococcal desoxyribonuclease. The fibrous material was then drained of alcohol, and redissolved in saline for repeated deproteinizations. The usual preparation received a total of 9 treatments with chloroform and amyl alcohol, and 3 alcohol precipitations. The final solutions in saline were clear and viscous, their volume being adjusted from 5 to 10 cc., according to the apparent bulk of the precipitates obtained in the course of preparation. These solutions contained from 0.13 to 0.35 mg. per cc. of desoxyribonucleic acid as estimated by the diphenylamine reaction. The extracts were sterilized by alcohol precipitation and stored as sterile saline solutions. The biological activity of these solutions was tested in transformation tests in which the final concentra-

tion of desoxyribonucleic acid was in the range of 0.65 to 1.75 microgram per cc. of broth. It has been shown that as little as 0.004 microgram per cc. of Type III transforming principle induces regular transformations of R pneumococci into SIII (2). Quantitative studies were not made with the crude preparations, but titrations showed that the amounts employed for testing were well in excess of the minimum amount of extract required to obtain transformation.

The Transformation Tests

In performing transformation tests, 2 cc. of broth containing the necessary accessory serum constituents, noted above, was pipetted into small sterile tubes. The broth, described previously (2), was adsorbed with charcoal prior to its use. Transformation tests were made in quadruplicate or quintuplicate, each tube containing the same amount of transforming principle. The latter was added to the tubes by diluting in saline a small sample of the stock solution of the transforming principle to be studied, so that 0.1 cc. contained the desired amount of desoxyribonucleate. A tenfold dilution of the stock solution was usually employed. In each experiment one or more control tubes were provided, to which no transforming principle was added. All tubes were inoculated with 0.05 cc. of a 10^{-4} dilution of a 4 to 8 hour blood-broth culture of the strain being transformed, unless otherwise noted. Transformation tubes and controls were incubated from 16 to 24 hours at 37°C. At this time preliminary readings could be made in certain of the transformation systems where the transformed bacteria and their progeny grew diffusely in the supernate. This is typical of the R to S transformations. Some of the transformations to be described do not give clear readings of this kind, since in some cases the transformed cells were also agglutinated by the same agent used to agglutinate the inoculated strain. In every experiment samples of the populations in each of the tubes were withdrawn for examination by streaking a loopful of culture upon a sector of an agar plate. Upon incubation, these samples produce about 100 to 200 well isolated colonies. Conclusions concerning the composition of the populations after transformation were based upon a study of the colonies in these subcultures.

Strains of Pneumococci Studied

In the present study the classical rough races will be called "R," while the classical smooth races will be referred to as "S." Since the S races can be of various serological types, the Roman numeral corresponding to the serological classification of the race will be added to classify the strain more precisely. For example, the Type III pneumococcus used in the present study will be referred to as SIII. Implicit throughout the present report will be the assumption that the salient difference between R and S races is the fact that the individual S bacterium possesses a specific transforming principle which is lacking in R pneumococci.

The following strains of pneumococci have been used in the course of the experiments to be discussed:—

1. Strain A66, a Type III smooth strain, used for preparation of Type III transforming principle. (Colonies of this strain are illustrated in Fig. 5.)

2. Strain R36A, a rough strain derived originally from a Type II smooth strain, D39. This strain has two characteristics of importance in transformation experiments. Bacteria of this strain can be transformed to any of several different types of smooth strain under the action of the corresponding transforming principles (4). Furthermore, no spontaneous reversions to the smooth condition have ever been observed to occur, although the strain has been repeatedly subjected to conditions favorable for the detection of such reversion. This strain has been used both as a source of transforming extracts and as a test organism for the transforming activity of various extracts. (Fig. 2.)

3. Strain ER, derived by spontaneous mutation from strain R36A. This variant has an extremely rough colony form, and in liquid culture grows in aggregates. It has been used

both as a source of desoxyribonucleate, and as a test organism for transforming activities of various extracts. (Fig. 1.)

4. Strain SIII-1, a mutant Type III pneumococcus which appeared spontaneously in an old refrigerated culture of an SIII line. This latter line had been established from a single colony of strain R36A transformed to the SIII condition, under the action of an SIII transforming principle isolated from strain A66. (Fig. 3.)

5. Strain SIII-2, a mutant Type III pneumococcus which appeared in a transformation experiment in which strain R36A was being transformed under the action of SIII transforming principle isolated from strain A66. (Fig. 4.)

These last two strains have also been used both as organisms for testing transforming activities of various extracts, and as sources of transforming principles.

Experiments with the ER Strain

Strain ER (extreme rough) can revert to the R condition. After a few serial passages of ER in ordinary liquid culture, appreciable numbers of R pneumococci may be found. On solid media populations of ER pneumococci appear to be completely stable. Thus, the instability of strain ER in liquid media can be attributed to selective forces favoring the growth of the diffusely growing R bacteria. Strain ER can, however, be maintained in pure condition in liquid culture if it is grown in a shallow layer of broth in an Erlenmeyer flask. Highly aerobic conditions are not favorable for the growth of pneumococci, and apparently growth in shallow layers is relatively more unfavorable for the diffusely growing R bacteria than for the ER bacteria, which presumably establish localized, less aerobic, areas. After cessation of growth in these shallow layer cultures, ER pneumococci must be transferred into small sterile test tubes for preservation, for like other pneumococci, these cannot be preserved under highly aerobic conditions.

The characteristic colonies of strain ER are shown in Fig. 1.

Transformation of ER to R.—Transformations of the ER strain were undertaken in the absence of added R agglutinins, since the ER bacteria grow spontaneously in an aggregated state. Serum factor in the form of bovine serum albumin was added to nutrient broth, as well as transforming principle obtained from SIII pneumococci. Experiments were carried out in the usual manner, using a volume of 2 cc. of medium placed in small test tubes. The tubes were inoculated with 0.05 cc. of a 10^{-3} dilution of an 8 hour culture of ER, grown in a shallow layer.

In this transformation system no SIII organisms were found after the usual incubation period. However, the populations of pneumococci in all the tubes which received the transforming principle were found to be predominantly R pneumococci. In an occasional control tube containing no added transforming principle, appreciable numbers of R pneumococci were also found. Since the ER pneumococcus can give rise spontaneously to R forms, two hypotheses could be offered to explain the invariable presence of the large numbers of R pneumococci in the tubes which had received the transforming principle. Either this desoxyribonucleic acid preparation was acting as a powerful selec-

tive agent for spontaneously formed R pneumococci, or this material was acting as a specific transforming agent as it is known to do in transformations of R pneumococci into SIII.

Experiments disclosed the following facts, and clarified the mode of action of the desoxyribonucleic acid fraction of the SIII bacteria upon the ER strain.

1. The predictable appearance of large numbers of R pneumococci in cultures of the ER strain depends upon the presence in the environment of adequate concentrations of the desoxyribonucleic acid fraction from suitable pneumococci. A titration of the transforming activity of this fraction of SIII pneumococci is shown in Table I. This same highly purified desoxyribonucleate has approximately the same degree of activity in changing ER populations into R as it has in changing R populations into SIII, provided titrations are performed in the appropriate environments, both using albumin as a source of serum factor.

TABLE I

Titration of the Activity of Type III Transforming Principle in Transforming Strain ER to R

Transforming preparation 55*	Quadruplicate cultures‡			
Amount added	1	2	3	4
micrograms				
6.0	R	R	R	R
1.9	R	R	R	R
0.6	R	R	R	R
0.19	ER only	R	R	R
0.06	R	Few R	Few R	Few R
None	ER only	ER only	ER only	ER only

* The stock solution of transforming agent contained 0.6 mg. per cc. of desoxyribonucleic acid, estimated by the diphenylamine reaction. 0.1 cc. of appropriate dilutions were added to tubes containing 2 cc. of broth to which albumin and pyrophosphate had been added Inoculum was 0.05 cc. of a 10^{-3} dilution of an 8 hour aerobic culture of strain ER.

‡ The populations were plated after 16 hours of incubation at 37°C. The symbol R indicates the presence of R colonies in addition to ER.

2. To obtain the regular production of large numbers of R pneumococci in the growing ER populations, it is necessary to add to the medium accessory serum factor (*e.g.* bovine serum albumin) as well as adequate amounts of the pneumococcal desoxyribonucleate.

3. Transformation of populations of ER pneumococci into R is a special property of certain desoxyribonucleate preparations. Active desoxyribonucleates are obtained from SIII and as well from R pneumococci (R36A). Highly polymerized desoxyribonucleates obtained from ER pneumococci, two

different strains of streptococci, and from calf thymus are totally inactive in this respect.

4. Small concentrations of the enzyme desoxyribonuclease (1 microgram per cc.) inhibit the action of active desoxyribonucleates in transforming populations of ER pneumococci into R, provided the enzyme is added to the transformation cultures prior to the end of the first 5 hours' incubation.

5. ER pneumococci grown for at least 5 hours in the albumin-broth mixture are capable of rapidly interacting with the transforming substance in such a way that desoxyribonuclease can no longer block the transformation into R. Hence, the ER strain undergoes the phenomenon called sensitization, described first in the studies of transformation of R pneumococci into S (6). This sensitized state, during which rapid interaction with the transforming principle is possible, is transitory for the ER pneumococci as had been found for the R, and disappears after the first 7 to 8 hours of incubation have been completed. The technique used to study the sensitization phenomenon in the transformations of the ER strain was essentially the same as described in analysis of the R to SIII transformations, and will not be discussed here.

These facts are in agreement with the interpretation that although ER pneumococci can mutate spontaneously and unpredictably to the R condition, predictable transformations are specifically induced under the action of desoxyribonucleates of certain pneumococci.

It should be noted that the same formal proof which existed in Griffith's transformation experiments (8) is not available for the case of the ER to R transformations. The essential point of the argument which established the existence of specifically induced transformation was the fact that while some R strains can spontaneously revert to the S condition, they never revert to a serological type heterologous with respect to their origin. Thus, the fact that some R strains not only could be transfotmed to S of heterologous type, but also to the type corresponding to the origin of the heat-killed cells used to induce the transformation, provided definitive evidence that the transformation was specifically induced. The ER strain can spontaneously revert to R, and it is the same R, as far as is known, that is produced as a consequence of induced transformation of the ER pneumococci. Furthermore, the transformation of ER into R is induced by more than one transforming extract, the desoxyribonucleate fractions of both R and SIII pneumococci being active. Hence the exclusive proof which established that the R to S transformation was specifically induced cannot be provided in the case of the transformation of ER pneumococci into R. However, the completely analogous requirements for both transformations, and the virtually identical mechanisms which operate in both cases, make it most probable that essentially the same phenomenon is occurring in both kinds of transformations; *i.e.*, that both changes are specifically induced by an agent which is apparently a specific desoxyribonucleate.

One difference exists between the conditions necessary for the transformation of R pneumococci into S, and ER pneumococci into R. In the latter transformation rough cell agglutinins are not only not required, but actually must be

omitted. This does not imply that the agglutinated state is not required for the ER pneumococci undergoing transformation, since, as has been mentioned above, the ER strain grows spontaneously in an aggregated condition. The addition of R cell agglutinins results in a more compact aggregation of the ER bacteria, but completely inhibits the appearance of R forms in tubes which otherwise would be expected to contain more than 50 per cent R pneumococci, produced as a result of transformation of the original ER population. No SIII pneumococci were formed directly from the ER strain treated with a desoxyribonucleate of SIII bacteria, in the presence of R agglutinins. Thus, like the other morphological mutants obtained from strain R36A (2), strain ER is "incompetent" to undergo direct transformation into the SIII condition.

Two-Step Transformation of Strain ER into R into SIII.—The R pneumococci, produced by transformation of the ER strain as described above, were competent to be transformed in their turn to SIII by means of the same desoxyribonucleate, prepared from SIII pneumococci, which had induced the transformation of ER pneumococci into R. Thus, starting with the ER strain, a two-step transformation could be performed, eventually yielding SIII pneumococci. The second step, performed in the presence of R agglutinins, could readily be accomplished using any of a number of clone lines of R forms isolated from transformation experiments with the ER strain.

A single difference exists in the external conditions required by the two steps. To transform strain ER into R, R agglutinins must be omitted, while to transform R pneumococci into S, the R agglutinins must be present. It seemed possible, therefore, that in the course of a single growth cycle of an ER culture and under the action of a single dose of SIII desoxyribonucleate the two steps might be achieved if the R agglutinins were added at the appropriate time. The transformation of strain ER into R is completed after the first 5 hours of incubation (see above). Hence, in a transformation of strain ER, R agglutinins were added to some of the tubes after a preliminary incubation of 5¾ hours. At the same time 2 cc. of fresh culture medium was added to prolong somewhat the growth of the cultures in the altered environment. A single addition of transforming principle obtained from SIII pneumococci had been made to all tubes at the time of inoculation with a drop of diluted ER culture. After 24 hours of incubation, a sample of the contents of each tube in the experiment was subcultured on agar plates. In all tubes to which the R agglutinins were added at 5¾ hours, ER, R, and SIII pneumococci were found in the final populations. In tubes not receiving the R agglutinins, only R and ER pneumococci were found. It was possible repeatedly to obtain thus the two transformation steps in a single culture of strain ER, and with a single portion of transforming principle, by supplying the R agglutinins at the proper time.

When strain ER was transformed under the action of a desoxyribonucleate obtained from the R strain R36A, the sole transformation products were R cells, regardless of the addition of the R agglutinins after 5¾ hours' incubation.

This is in agreement with the previously reported findings that this R strain contains no transforming principle inducing synthesis of specific polysaccharide (3). It therefore appears that the desoxyribonucleate fractions of both R and SIII pneumococci have one transforming principle in common, that which transforms strain ER into R, while this fraction of the SIII pneumococci contains a second transforming principle as well, that which transforms R pneumococci into SIII. The analogous fraction of strain ER pneumococci has neither transforming activity, and presumably neither of the two transforming principles which appear to exist in the desoxyribonucleate fraction of the SIII organisms.

There remains to be explained why the transforming principle found uniquely in the Type III pneumococcus does not appear to act directly upon ER pneumococci. There is as yet no evidence that this transforming principle can propagate in the absence of the transforming principle found common to both SIII and R pneumococci. This latter principle, which for brevity can be called the R transforming principle, can on the other hand be propagated in the absence of the SIII transforming principle, for that is its condition in the rough strain used for these transformation studies. Thus, the mutually independent existence of these two transforming principles as genetic units is not demonstrated by the reported experiments. It is possible that the action of the SIII transforming principle upon the pneumococcal cell is dependent upon the phenotypic activities of the R transforming principle. Until this is shown to be the case, or until pneumococci are found which possess the S transforming principle in the absence of the R principle, strict proof that the desoxyribonucleic acid fraction of SIII pneumococcus contains at least two distinct entities controlling hereditary traits cannot be offered.

Transforming Principles Present in the Transformed Pneumococci.—Each step of the two-step transformation just described produced pneumococci which were permanently altered with respect to the newly acquired character. From R pneumococci obtained by transformation of ER forms, and from the stepwise produced SIII pneumococci, active transforming principles were isolated. The transforming extracts of the R strain produced by transformation of the ER strain possessed only the capacity to transform ER pneumococci into R. The transforming extracts of the SIII cells produced by two-step transformation of ER possessed both the capacity to transform ER pneumococci into R, and R into SIII. Hence, the properties of the transforming extracts of the doubly transformed pneumococci reflected both steps of the two-step transformation, and the pneumococci thus produced appeared to have acquired two transforming principles in the course of the two transformation steps.

The Intermediate SIII Strains

Between the colony forms S and R there exists a spectrum of gradations. Colonies having an appearance intermediate between R and S are produced

by stable strains of pneumococci which agglutinate to varying degrees in both type-specific and group-specific antisera (9, 5). Usually S pneumococci are not agglutinated by group-specific antisera unless steps have been taken to remove the polysaccharide capsule. The intermediate forms have rarely been found to be virulent, but they have been found to be capable of reverting to the virulent S condition. Such intermediate forms have been obtained by growing smooth pneumococci in homologous specific antiserum. Very commonly, however, intermediate forms are encountered in old, refrigerated S cultures, indicating that the antiserum is not essential for their production.

The mutant SIII strains to be discussed are two of a number of spontaneously appearing variant SIII pneumococci which have been encountered over a period of 2 years' work. Their origin has been noted above.

Strain SIII-2 produces a mucoid colony on solid medium, but the colony is distinct from the mucoid colonies of the SIII strain, A66, and of R pneumococci transformed by the SIII transforming principle isolated from strain A66. The colony of SIII-2 is smaller, dries out more rapidly, and autolyzes sooner than do the colonies of the latter two strains. In general the colonies of the SIII strains isolated from nature are more mucoid than those of other smooth types. The strain SIII-2 thus resembles superficially other smooth races of pneumococci more than it does the parent SIII line from which it was indirectly derived. The variant is, however, serologically a Type III organism, and gives a quellung reaction (swelling of the capsule) in Type III antiserum. SIII-2 is capable of establishing infection when relatively small numbers of organisms are injected intraperitoneally into mice. See Table II. The strain is stable in liquid culture, but is capable of giving rise to rare mutants which produce colonies like those of the normally mucoid SIII strains.

Strain SIII-1 is not mucoid, and its colony can be distinguished, with difficulty, from an R colony. Although by agglutination tests SIII-1 must be classified as a Type III organism, it does not give a quellung reaction in Type III antiserum. Nor is strain SIII-1 virulent, although it is capable of reverting to the normally mucoid, virulent condition. See Table II. The SIII-1 variant is analogous to the variant of an SII strain described by MacLeod and Krauss (5).

Colonies of these strains are shown in Figs. 3 and 4.

Serological Characterization of the Variants.—The various races of S pneumococci are differentiated by their capsular antigens, the specificity of which depends upon the chemical composition of the capsular polysaccharides. However, S pneumococci as well as R pneumococci possess a large number of somatic antigens which appear to be common to the group Pneumococcus. Thus, a distinction is made between the group-specific antigens common to encapsulated and non-encapsulated forms alike, and the type-specific antigens, found uniquely in the encapsulated races. As far as is known, each S race forms only one kind of capsular polysaccharide, or type-specific antigen.

Antisera resulting from the injection of R pneumococci into animals possess antibodies directed against the group-specific antigens, while antisera against S forms usually contain antibodies which react with both group-specific and type-specific antigens. Thus, an antiserum prepared with an SIII strain is usually capable of agglutinating both R and SIII strains, but an antiserum prepared with an R race is incapable of agglutinating the usual S races. An antiserum containing type-specific antibody only, which reacts only with homologous polysaccharide or organisms, can be made by adsorption of a given antiserum with heat-killed R pneumococci.

Using antisera made with normal Type III pneumococci, and antisera against strain R36A, an analysis was made of the antigenic structure of the SIII races

TABLE II

Results of Intraperitoneal Injection of Mice with 8 Hour Blood-Broth Cultures of the Intermediate Smooth Strains

Infective dose	Strain SIII-2		Infective dose	Strain SIII-1	
	Result	Organism recovered		Result	Organism recovered
cc.			*cc.*		
10^{-1}	D 30 hrs.	SIII-2	0.5	D 24 hrs.	SIII-1
10^{-2}	D 24 hrs.	SIII-2	0.5	D 24 hrs.	SIII-1
10^{-3}	S		0.5	D 48 hrs.	SIII-N
10^{-4}	D 36 hrs.	SIII-2	0.5	S	
10^{-5}	S		0.5	S	
10^{-6}	D 36 hrs.	SIII-2	0.5	S	

D = dead.
S = survived.

used in the present study. The essential points shown by these studies are the following:—

1. The normal SIII strain, to be referred to henceforth as SIII-N, and mutant strain SIII-2 are agglutinated only by antiserum containing antibody reacting with the Type III polysaccharide (Tables III and V).

2. Strain SIII-1, the non-mucoid variant, and also strain R36A, an R strain, agglutinate in antisera containing group-specific antibody (Table V). Larger quantities of antibody are required to agglutinate SIII-1, than are required to agglutinate the R strain.

3. Strain SIII-1 is also agglutinated by Type III type-specific antibody. This reaction is revealed in Type III antipneumococcal serum adsorbed with R bacteria to remove the group-specific antibodies normally present in such sera (Table IV).

4. Less Type III antibody is required to agglutinate intermediate strains SIII-1 and SIII-2, than is required to agglutinate the fully mucoid SIII-N strain (Tables III and IV).

5. When strains SIII-1, SIII-2, and SIII-N are grown in the presence of group-specific antibody and the enzyme which hydrolyzes the Type III capsular polysaccharide, it is found that the enzyme uncovers the group-specific antigens of all three strains. Strains-SIII-N and SIII-2, which normally agglutinate only in the presence of adequate amounts of Type III antibody, are so altered by the enzyme that they can be agglutinated by R antibody, or group-specific antibody. Similarly, through the action of the enzyme, SIII-1 pneumococci are rendered sensitive to much smaller amounts of R antibody than are otherwise required to agglutinate this strain. Thus, the enzyme which splits

TABLE III

Thread Test Reactions of SIII-N Cells and SIII-2 Cells in Gamma Globulin Fraction of Type III Immune Horse Serum

Antigen	Dilution of antibody solution in broth									
	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120
SIII-N	++++	+++	++	++	+	–	–	–	–	–
SIII-2	++++	++++	++++	+++	+++	+++	++	+	+	–

Serum fraction contained rough agglutinins but neither strain reacted with rabbit R agglutinins (*cf.* Table V). ++++, complete sedimentation of growth. +++, slightly granular supernate. ++, bulk of growth in granular supernate. +, finely granular supernate, no sedimented growth.

TABLE IV

Agglutination Reactions of SIII-N and SIII-1 Cells in Type III Immune Rabbit Serum

Antigen	Dilution of antibody solution in 0.85% sodium chloride										
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048
SIII-N	++++	++++	++++	+++	+++	+++	++	+	–	–	–
SIII-1	++++	++++	++++	+++	+++	+++	+++	+++	+++	++	–
R36A	+	+	–	–	–	–	–	–	–	–	–

Serum absorbed with R36A to remove rough cell agglutinins. Read as in Table III.

the Type III polysaccharide attacks also the type-specific antigens of the mutant SIII strains (Table V).

These experiments indicate that the mutant strains each form Type III polysaccharide. It should be emphasized that while serological cross-reactions are found between a number of polysaccharides of pneumococcus, none of the polysaccharides which show serological cross-reactions with the Type III polysaccharide can be split by the enzyme used to hydrolyze this latter polysaccharide in the present experiments. Thus, the data just summarized support the view that the polysaccharide formed by the SIII mutants is unaltered in chemical composition from that formed by the normal Type III strain.

Two rabbits were injected with living SIII-1 cells from ½ cc. of an 8 hour

blood-broth culture, on 4 successive days during each of 2 weeks. 7 days following the last injections two sera were obtained which reacted down to concentrations of 1:64,000 and 1:16,000 of purified Type III polysaccharide respectively.

Attempts were made to adsorb the type-specific antibody from a Type III immune serum by means of cells of the non-mucoid variant. 4 cc. of a diluted serum was adsorbed with the variant cells obtained from 1500 cc. of culture.

TABLE V

Thread Tests with the Three SIII Strains, in Serial Dilutions of a Concentrated Gamma Globulin Fraction of Serum from Rabbits Immunized with R Strain R36A

Series A

Antigen	Dilution of antibody solution in broth							
	1/40	1/80	1/160	1/320	1/640	1/1280	1/2660	1/5120
SIII-N	–	–	–	–	–	–	–	–
SIII-2	+	–	–	–	–	–	–	–
SIII-1	+++	+++	+++	+++	++	++	+	–
R36A	++++	++++	++++	++++	++++	++++	++++	++++

Series B

Antigen		Dilution of antibody solution in broth					
		1/250	1/500	1/1000	1/2000	1/4000	1/8000
SIII-N	Enzyme-hydrolyzing SIII polysaccharide present in constant concentration	+++	+++	++	++	++	
SIII-2			++++	++++	+++	++	
SIII-1		++++	++++	++++	++++	++++	
R36A		++++	++++	++++	++++	++++	++++

In series A, antibody solution was diluted with broth. In series B, antibody solution was diluted with broth containing the enzyme which hydrolyzes Type III polysaccharide. Read as in Table III.

As a result of this adsorption a fourfold decrease was observed in the agglutination titer of this serum with normal SIII cells. The SIII-1 cells had removed some of the Type III agglutinins from the serum, but left behind considerable activity. The residual specific antibody, like the unadsorbed antibody, agglutinated SIII-1 bacteria at higher dilutions of antiserum than it did the normal SIII cells, although the antibody removed had been adsorbed by the antigens of SIII-1. Repeated adsorptions led to the same result. The R antibody was completely removed, showing both that the non-mucoid variant SIII could adsorb R antibody, and that the antibody left behind was solely type-specific antibody. It can thus be concluded that the strain SIII-1 adsorbs very little of the type-specific antibody, and that repeated adsorption does not

result in a qualitative change in the type-specific agglutinins remaining in the antiserum. There is no indication from these experiments that the antigen of the SIII-1 pneumococcus differs qualitatively from the antigen of the SIII-N strains.

The purification and study of the polysaccharides of the mutant SIII strains will be necessary before it can be definitely stated that normal and mutant strains are producing exactly the same polysaccharide. The data thus far obtained are in harmony with the view that each of the organisms studied is capable of synthesizing the Type III polysaccharide, and that the differences between the strains lie in the rates at which the polysaccharide is formed or released at the surfaces of the bacteria. The obvious morphological differences between the colonies of the SIII strains suggest that the quantity of polysaccharide is less in strains SIII-2 and SIII-1 than in the normal SIII strains. There are two other reasons for believing that the differences between the SIII strains are quantitative.

1. It is clear that SIII-2, SIII-1, and SIII-N strains are agglutinated by different quantities of Type III antibody. The non-mucoid SIII-1 strain requires the smallest amount, SIII-2 requires more, while SIII-N requires the most. Since the Type III polysaccharide is water-soluble, antibody is neutralized by polysaccharide in solution around the bacteria, and hence is neutralized without contributing to the agglutination of the bacteria. Thus, the strain releasing the smallest amount of polysaccharide in solution would be expected to be agglutinated by the smallest quantity of specific antibody.

2. The three strains differ with respect to the ease with which the same concentration of polysaccharide-splitting enzyme can free the bacterial surfaces of specific polysaccharide (Table V). Although the enzyme was prepared by adapting *B. palustris* so that it could utilize as a carbon source the capsular polysaccharide of the normal Type III pneumococcus, the enzyme is more effective in uncovering the non-specific antigens during growth of strains SIII-1 and SIII-2 than in uncovering these antigens in cultures of the normal SIII strain. This hydrolytic enzyme does not destroy the capacity of the pneumococcal cell to form Type III polysaccharide, and whether or not the non-specific surface antigens are uncovered during growth in the presence of the enzyme depends very likely upon the balance existing between the rate of destruction of the polysaccharide and the rate of its synthesis or release at the bacterial surface.

The Nature of the Altered Heredity of SIII-2 and SIII-1.—The SIII-N bacterium differs from the R in possessing the Type III transforming principle, which will be referred to as the SIII-N transforming principle. The question was therefore raised whether the mutant SIII strains possessed the same transforming principle or some analogous transforming principle.

Extracts possessing transforming activity were obtained from both of the intermediate smooth strains. With these extracts the susceptible R strain

R36A could be transformed to the SIII-2 or the SIII-1 condition according to the origin of the extract used. Each extract conferred upon the R pneumococcus the ability to synthesize the type-specific antigen, and as well, determined that the transformed cell formed the antigen in a particular altered condition. In no case did either extract transform R bacteria to the fully mucoid condition, nor was it ever observed that an extract from SIII-2 transformed R36A to SIII-1, or *vice versa*. Each extract possessed a strictly specific transforming action. The intermediate Type II strain studied by MacLeod and Krauss (5) likewise appears to contain a transforming agent which transforms R pneumococci into the intermediate Type II state. The results of these authors are analogous with the results just reported here.

A highly purified desoxyribonucleate was prepared from SIII-1. The extensive purification did not alter qualitatively its transforming activity. This desoxyribonucleate fraction transformed R36A to SIII-1 and also transformed strain ER into R. Hence, like the SIII-N nucleic acid it appeared to possess two transforming principles, each having a specific activity.

Rough strains were derived from the mutant strains SIII-1 and SIII-2 by serial passage of these strains in 10 per cent Type III immune serum. Both of these rough strains proved capable of being transformed to SIII-N under the action of the SIII-N transforming principle. This indicated that the hereditary properties of the mutant SIII strains permitted normal antigen synthesis provided the normal SIII transforming principle was possessed by the bacteria. This fact taken in conjunction with the isolation of transforming agents having altered activity indicates that it is the SIII-N transforming principle itself which has undergone some alteration in the mutant S strains.

Although phenotypically the SIII strains appear to differ from each other in a quantitative way, no evidence was found to indicate that the apparently quantitative variation of capsular polysaccharide formation was due to a variation of the quantity of SIII-N transforming principle, common to all of the SIII strains but present in different amounts. This may be seen from the following considerations.

If the variations in polysaccharide synthesis were due to the presence of different numbers of units of the Type III transforming principle in the various races, one would expect transformations of the R strain by means of the SIII-N transforming principle to yield a spectrum of Type III forms. Detailed examination of the kinds of smooth bacteria produced by such transformations showed that almost invariably normal Type III bacteria were produced. As a very rare exception this transforming principle will induce the production of variant smooth pneumococci. These anomalies have been of two sorts. Several lines of SIII pneumococci have been recovered, each characterized by apparent quantitative modification of polysaccharide synthesis. Strain SIII-2 is one such line. In addition, two different lines giving smooth colonies have been found which could not be typed by means of antisera against the first 33 types of smooth pneumococci. These deviations in the results of transformations are extremely rare, however, and have the same degree of unpredictability as has the phenomenon of mutation. Thus, it is concluded that just as no unit comparable to the normal Type III transforming principle exists in the desoxyribonucleic acid fractions

of the intermediate SIII strains, likewise no units comparable to the mutated Type III transforming principles seem to exist in the desoxyribonucleic acid fraction of the normal SIII pneumococci.

This conclusion is strengthened by the results of the following experiments. R pneumococci were transformed under the action of mixtures of equal amounts of the SIII-N transforming principle and either one of the mutant SIII transforming principles. Under these conditions, smooth strains corresponding to each of the transforming principles were recovered simultaneously in the transformed populations. Quantitative studies of the proportions of each kind of smooth pneumococcus strain were not made, because of the difficulties in assessing the rôle of selection in determining the final composition of the populations. However, the proportions recovered were approximately equal, when approximately equal amounts of each transforming agent were introduced. The results of these experiments can most simply be interpreted by supposing that each of the SIII transforming principles is a distinctive entity equally effective in competing in reactions with the sensitized R pneumococci.

Additive Effect of the Mutant SIII Transforming Principles

Each SIII transforming principle proved to be strictly specific in its action upon the R pneumococcus when it was introduced singly into the transforming system, or in experiments where the SIII-N principle was introduced simultaneously into the transforming system with either one of the mutant principles. However, when mixtures of the two mutant principles, SIII-1 and SIII-2, each in excess of the amount necessary to produce transformations, were introduced into the system for transforming R pneumococci, not two but three transformation products were recovered. The final population was found to contain both kinds of mutant SIII bacteria, and also normal SIII-N bacteria. Few of these SIII-N pneumococci were found, but their appearance is significant in view of the failure to obtain any such forms when the mutant transforming principles were added singly to this transforming system. The appearance of the SIII-N bacteria indicates that some form of summation can take place between the two mutant principles in their action upon a sensitized R pneumococcus, resulting in normal polysaccharide synthesis.

Nonetheless, it is clear that a simple quantitative difference between the SIII transforming principles does not exist. The SIII-2 agent used in excess of the amount necessary for transformation never yielded this summation; nor did the SIII-1 transforming principle. The existence of the interaction just described is, therefore, evidence that each of the mutated principles differs from the SIII-N principle in some unique respect.

Two-Step Transformations of R into SIII-1, into SIII-N

A rough pneumococcus which has been transformed to the lowest degree of smoothness by means of the principle obtained from SIII-1 bacteria can again be transformed, under the action of the SIII-N principle in an appropriate environment. SIII-1 pneumococci are poorly agglutinated by R agglutinins, and to transform them, it was necessary to provide a better means of agglutina-

tion. The techniques used have been described in detail above. Briefly, agglutination was achieved either by adding to the usual transforming system containing R antibody the enzyme which hydrolyzes the Type III polysaccharide, or by replacing the R agglutinins with SIII agglutinins. Although these techniques yielded completely agglutinated growth of the SIII-1 inoculum, the SIII-N cells produced by transformation had a tendency to escape the agglutinating mechanism. Thus, in tubes in which transformation occurred, the supernatant medium often contained granular or even diffuse growth.

In these systems, massive and regular transformations of SIII-1 populations to SIII-N were obtained. However, since the SIII-1 cells possess the capacity to mutate spontaneously both to the SIII-N condition and the R condition, three mechanisms may be conceived by which the SIII-N pneumococci could be formed. First, they could be formed by directly induced change of SIII-1 bacteria into SIII-N through the action of the SIII-N transforming principle. Second, they could appear through the selection of spontaneously occurring SIII-N mutants. Third, they could be produced by a transformation of the rare R mutants of the SIII-1 strain, through the action of the SIII-N transforming principle. Experiments were therefore done to determine how the SIII-N cells were produced in this transforming system. The following facts were ascertained:—

1. The regular appearance of SIII-N pneumococci is dependent upon the presence of adequate quantities of the SIII-N transforming principle. Titrations of the action of this purified desoxyribonucleate fraction of SIII-N pneumococci upon the SIII-1 population indicated that its activity was of the same general magnitude in this transformation and in transformations of R into SIII-N. In general, transformations of SIII-1 pneumococci into SIII-N occurred at slightly lower concentrations of a given preparation of SIII-N transforming principle than was needed for transformations of R into SIII-N. The significance of this greater sensitivity of the SIII-1 bacteria in the transforming system is not understood at present. Table VI shows a titration of an SIII-N transforming principle in both transformation systems. Both titrations were performed the same day, and using the same reagents. This pair of titrations shows an extreme manifestation of the differences in activity of the same desoxyribonucleate fraction in the two transformation systems.

2. Spontaneous mutation of SIII-1 to SIII-N has a negligible rôle in these experiments. Of 66 control tubes which did not receive transforming principle, only 1 was found to contain enough SIII-N pneumococci to be manifest upon plating.

3. The accessory serum factor is essential to obtain the regular and massive changes characteristic of the transformations of SIII-1 populations into SIII-N.

4. By use of the enzyme desoxyribonuclease it was found that in the transformation of SIII-1 into SIII-N the sensitization phenomenon occurred in a fashion exactly parallel to that already observed for strains R36A and ER.

The sensitization of SIII-1 pneumococci is achieved after a period of 4½ to 5 hours' incubation. After this time an exposure of as little as 5 minutes to the transforming principle is adequate to induce massive changes of the SIII-1 population into SIII-N.

The high degree of control over the outcome of the transformations of SIII-1 pneumococci into SIII-N precluded the possibility that rare spontaneous mutation followed by selection played a critical rôle in changing the SIII-1 population into SIII-N. However, it could be excluded on experimental grounds that the appearance of the SIII-N bacteria in the treated populations of SIII-1 bacteria was due to the transformation of rare R variants in the SIII-1 strain. It was observed many years ago that type-specific antisera inhibit the transformation of R36A into SIII-N (McCarty and Avery[1]). This inhibitory action is not confined to Type III antisera. In the present experiments, complete inhibition of transformation of R strain R36A into SIII-N was obtained with

TABLE VI

Comparative Titrations of the Activity of Desoxyribonucleic Acid Fraction of Type III Pneumococcus in Transforming R Cells into SIII-N and SIII-1 Cells into SIII-N

Transforming Preparation 55	Quadruplicate tests*			
Micrograms added	Inoculum—R36A		Agglutinins—R antibodies	
	1	2	3	4
6.0	SIII-N	SIII-N	SIII-N	SIII-N
1.9	SIII-N	SIII-N	SIII-N	SIII-N
0.6	R	R	R	SIII-N
None	R	R	R	R
Transforming Preparation 55	Inoculum—SIII-1		Agglutinins—SIII antibodies	
	1	2	3	4
0.6	SIII-N	SIII-N	SIII-N	SIII-N
0.06	SIII-N	SIII-N	SIII-N	SIII-N
0.006	Few SIII-N	SIII-N	Few SIII-N	SIII-N
0.002	Few SIII-N	Few SIII-N	Few SIII-N	No SIII-N
None	SIII-1	SIII-1	SIII-1	SIII-1

Dilutions of transforming principle were added in 0.1 cc. amounts to tubes containing 2 cc. of broth to which albumin and pyrophosphate and agglutinins had been added. Inoculum was 0.05 cc. of a 10^{-4} dilution of a 4 hour blood-broth culture of the strain designated.

* The populations were plated after 23 hours of incubation at 37°C.

[1] Personal communication.

the same solution of Type III antibody which provided essential environmental conditions for the transformation of SIII-1 into SIII-N.

These experiments have led to the conclusion that SIII-1 bacteria can be directly transformed into the SIII-N condition through the action of the SIII-N transforming principle, under the appropriate environmental conditions. While the *in vivo* experiments of MacLeod and Krauss (5) have suggested that a Type II intermediate form could be transformed into a normal Type II organism under the action of a normal Type II transforming principle, the evidence offered by these workers does not appear adequate to prove that the intermediate form itself was the organism undergoing transformation. A possible rôle of R pneumococci, formed by spontaneous mutation of the intermediate strain, might be adduced to explain the results of the cited authors. The *in vitro* techniques used in the present study upon the SIII races provide evidence which rather clearly indicates that a pneumococcal cell, already endowed with the capacity to synthesize a specific polysaccharide, can be transformed under the action of a transforming principle inducing the formation of a different (or more of the same) specific polysaccharide.

The SIII-1 pneumococcus could also be transformed to a Type II pneumococcus by means of a desoxyribonucleate fraction of Type II pneumococci. Studies upon these transformations will be reported in detail at some future date.

When the R pneumococcus was transformed first to the SIII-2 condition, further transformation to the SIII-N condition under the action of the SIII-N transforming principle was not found possible. Likewise, when the R pneumococcus was transformed first to the SIII-N condition, the SIII-N forms produced could be transformed neither to the SIII-2 nor to the SIII-1 condition. The SIII-2 and SIII-N principles induce the formation of large amounts of capsular polysaccharide, which may interfere with the sensitization process. This possibility should be kept in mind, as well as the possibility that the failure to obtain these transformations is due to some properties of the transformation principles themselves.

Mechanism of the Two-Step Transformation, R into SIII-1, into SIII-N.—The SIII-N bacterium produced by this two-step transformation has reacted with two distinct transforming agents. An investigation was therefore made to see whether the desoxyribonucleate fraction of this doubly transformed bacterium contained both the SIII-1 and SIII-N transforming principles.

Special techniques were employed to detect SIII-1 pneumococci in the final populations of these transformation experiments, since the SIII-1 colony is difficult to distinguish from an R colony. When cultures are made by mixing 100 to 200 pneumococci in liquid nutrient agar, just before pouring the plate, plates are obtained in which colonies are well separated and submerged. If blood is omitted from the agar, and a potent homologous type-specific antiserum added before plating, around each smooth colony a halo will appear after 48 hours of incubation. This halo results from the reaction between the diffusing soluble capsular polysaccharide and the type-specific antibodies in the agar. R pneumococci produce no halo. SIII-N

pneumococci produce halos several millimeters in diameter, which stand well out from the colonies. Between the ring of precipitate and the colony is a zone of clear agar, since the large amount of polysaccharide produced by this strain redissolves the polysaccharide-protein precipitate (prozone phenomenon). This halo travels centrifugally as incubation is prolonged. SIII-1 pneumococci, however, form small halos which show no discontinuity between the precipitate and the colony. By plating suitable dilutions of the populations produced by transformation of strain R36A with the transforming extracts described in the following paragraph, the population could readily be examined for the presence of SIII-1 pneumococci.

Nucleic acid extracts were made from the progeny of a single colony isolate of an SIII-N pneumococcus produced by two-step transformation of strain R36A. Also, an extract was made from the progeny of a mixed culture established from a large number of SIII-N colonies obtained directly from the plated population of one of these two-step transformations. With both preparations results were identical, and when their action on strain R36A was studied, only SIII-N and R colonies were found on the plates made from the final population. There was, thus, no indication that these extracts could transform strain R36A into SIII-1.

The SIII-N strains produced by two-step transformation of R show no signs therefore of having once been in contact with and transformed by the SIII-1 transforming principle. It is as though in the two-step transformation of R into SIII-1 into SIII-N, the SIII-1 transforming principle has been, in effect, destroyed by the second transformation step. Hence the second step of this two-step transformation may consist of an exchange of the SIII-1 transforming principle in the bacterium with the SIII-N principle in the environment; or the second step may consist of some kind of interaction between the two principles, in which the SIII-1 principle loses its independent existence.

Analysis of the Specificity of the SIII-2 Transforming Principle in the Two-Step Transformation of R into SIII-1 into SIII-2.—Two-step transformations could be done by inducing the transformation of R pneumococci into SIII-1, by means of the appropriate principle, and following this, transforming the SIII-1 pneumococci under the action of the SIII-2 transforming principle. As has been indicated, two distinct transformation systems are necessary for the two steps, since SIII-1 pneumococci are poorly agglutinated by R antibody.

At the end of the second step, the final population of pneumococci found in the tubes which had received the SIII-2 transforming principle was composed of three kinds of pneumococcal cells. First, there was the residue of untransformed SIII-1 bacteria; second, there were large numbers of SIII-2 bacteria; third, there were small numbers of SIII-N forms. In the control tubes, receiving no transforming principle, only SIII-1 forms were found. Thus, under the action of the SIII-2 transforming principle, two transformation products were produced from the SIII-1 bacteria inoculated.

It should be emphasized that the SIII-2 transforming principle, acting upon R pneumococci, induces the formation of SIII-2 pneumococci only. Thus, here, as in the experiments cited above in which the SIII-1 and SIII-2 trans-

forming agents were introduced simultaneously into the transformation system of strain R36A, it appears that some form of summation between the two mutant principles can occur if the SIII-2 principle is interacting with a pneumococcus already possessing the SIII-1 principle. Summation does not always occur between these two principles. This is clear from the fact that the majority of new kinds of pneumococci produced in the transformation of the SIII-1 forms by the SIII-2 principle are SIII-2 forms.

This effect of the SIII-2 principle, when acting upon the SIII-1 pneumococcus, suggests again that transformation can be either an exchange reaction (producing SIII-2 forms), or some form of interaction (producing SIII-N forms) with the SIII-1 principle already possessed by the bacterium undergoing the transformation.

DISCUSSION

Transforming activity may be regarded simply as a new kind of character and it is possible that the transforming agents themselves are better suited to elucidate bacterial heredity than the more immediately accessible characters of bacterial cells, such as enzymatic constitution or antigenic structure. Transforming agents appear to be concerned with the heredity of pneumococcus in much the same fashion that genes are concerned with the heredity of higher organisms. Thus, they appear to have autocatalytic and heterocatalytic functions, and play a decisive rôle in determining the properties of a given pneumococcus and its progeny. It is, of course, not clear whether a transforming principle is a complete genetic determinant, since the transformed cell contributes the necessary environment for the expression of its activity. However, it is clear in the case of the R into SIII transformations that the transforming extracts include the constituent of the genetic system which is essential to the synthesis of a capsular polysaccharide, and which determines the chemical nature of this polysaccharide. Strictly speaking, very little more can be said even of genes with reference to their relationship to the characters they determine.

Certain aspects of the present experiments suggest further that the transformation technique may, in fact, be generally applicable to the study of the mechanism of heredity in the pneumococcus. The following points may be cited:—

1. The discovery of a new transforming principle, active in transforming ER cells into R, provides evidence that more than one character of the bacterium is under the control of a transforming principle having the properties of a desoxyribonucleate.

2. Evidence presented indicates that the desoxyribonucleate fraction of the SIII bacterium contains a minimum of two transforming principles. Even though complete evidence has not yet been obtained to the effect that the two transforming activities possessed by this fraction can be ascribed to two com-

pletely independent genetic units, such an assumption remains the most plausible.

3. Application of transformation techniques in a study of two mutated races of SIII pneumococcus showed that the SIII transforming principle itself had mutated in these races, giving rise to the mutant properties of these strains. Mutation of other hereditary factors in the altered strains which might result in modified polysaccharide synthesis, could be ruled out. Thus, by the transformation study it has been possible for the first time to determine that a mutated bacterium differs from its normal progenitor because of a spontaneous alteration which has occurred in a given entity possessing genetic activity. Further, it could be seen that it was the same entity which had undergone mutation in each of the mutated races.

The present study differs from preceding ones because the mutation of the SIII-N strain produced forms in which a mutated SIII transforming principle could be demonstrated. R forms also may contain a mutated S transforming principle, the heterocatalytic activity of which is not obvious.

4. The R transforming principle which induces the ER to R transformation is found in the desoxyribonucleate fractions of both the R and S bacteria thus far studied. Hence, although the R-ness of the S pneumococcus is not visible in its colony morphology, it can be demonstrated that the R principle is nonetheless present in the S pneumococcus. This kind of information about the genetic constitution of the S bacterium is analogous to that obtained in classical genetics when the experimenter demonstrates the presence of genes whose phenotypic manifestations are in some way masked.

This last consideration leads to a problem of great importance in any study of hereditary systems. It is known that a gene may be masked by a dominant allele or by non-allelic genes. If transforming principles are genetic determinants, how can their relations to each other be assessed? For example, are the R and S transforming principles allelic or not?

Clearly, if allelism were to be defined strictly in terms of the techniques usually employed to discern it, allelism cannot be demonstrated in asexual organisms. If, however, it be granted that the absence of crossing-over between two genes is a secondary aspect of allelism, and that the primary property of allelism consists of the alleles having been derived from common gene ancestors, it is justifiable to consider as alleles all genetic determinants fullfilling this primary condition, even in asexual forms. It is perhaps simpler to refer to such genetic units as homologous. The problem remains how to assess the relationships between genetic units, in the absence of sexual fusion.

In the body of the present paper, observations were set forth which led to the conclusion that the SIII-N, SIII-2, and SIII-1 transforming principles are distinct, qualitatively different entities, the latter two of which have arisen by spontaneous mutation of the former. Granting that this evidence is sufficient, it is justifiable to look upon these transforming principles as homologous genetic

elements—or alleles, if one is content to define alleles in terms of their lineage.

It appears thus far that a given race of pneumococcus can possess at a given time only one (or none) of the SIII principles. This is particularly in evidence in the two-step transformation of R into SIII-1, SIII-1 into SIII-N. Here it is found that in the second transformation step the SIII-N principle replaces the SIII-1 principle which was readily demonstrated in the desoxyribonucleate fractions of the SIII-1 pneumococci produced by the first transformation step. This is in contrast with the simultaneous occurrence of both R and S principles in the SIII forms thus far studied. Likewise, in the two-step transformation of ER into R, R into SIII-N, the doubly transformed cell can be shown to have acquired both the R and SIII principles. This R principle has been found in the SIII races and as well in the R strain R36A, derived originally from a Type II culture of S pneumococci. Thus, there is no indication that the R principle and the SIII principles have been derived from a common ancestor-transforming principle.

From these facts it is possible at present to visualize how a distinction can be made between genetically related (homologous, allelic) transforming principles and genetically unrelated ones by means of the transformation technique. It may very well be that in successive transformations with genetically related principles, transformation consists of a replacement or interaction of the first introduced principle with the second. On the other hand, after successive transformations with two genetically unrelated principles both principles are demonstrable in the desoxyribonucleate fraction of the doubly transformed organism.

It is implicit in this hypothesis that a condition analogous to heterozygosity does not exist with respect to the transforming principles of a pneumococcus. This appears, in fact, to be the case with the SIII principles.

The SIII principles and the R principle have been referred to here as entities. This is justified by the fact that there does not seem to be any quantitative or qualitative variation in the action of any one of these transforming principles upon the appropriate pneumococcal race, except as a rare deviation. This indicates that the induction of polysaccharide synthesis, for example, results from an interaction between the susceptible R form and a qualitatively distinct unit in the desoxyribonucleate fraction of the S organisms. There is no way of knowing at present how many particles in the desoxyribonucleate solution compose each biologically complete entity. With respect to the SIII transforming principles, the experiments reported above make it highly unlikely that they differ from each other by being composed of different numbers of particles, n of which must be possessed in order to produce the SIII-N phenotype. However, the existence of an interaction between the SIII-2 and the SIII-1 transforming principles reacting with a given pneumococcus to produce the SIII-N form, remains to be explained. This phenomenon could most

simply be understood if the SIII-N transforming principle could be subdivided into two biologically active agents, having respectively SIII-2 and SIII-1 transforming activity. No evidence could be found that the SIII-N transforming principle is such a biparticulate entity. It is likely that to understand these experiments we will have to understand at what level of molecular organization genetic activity makes its appearance, and to what extent the very large molecules present in the transforming extracts behave as indivisible units in cell metabolism.

I am grateful to Dr. Maclyn McCarty for preparations of the enzyme desoxyribonuclease, the two streptococcal desoxyribonucleates, and the thymus desoxyribonucleate. It is a pleasure also to acknowledge the many helpful discussions of this work with Dr. O. T. Avery, Dr. McCarty, and Dr. R. D. Hotchkiss; and, in preparation of the manuscript, the extensive discussions of the genetic aspects of the transformation phenomenon, with Professor Boris Ephrussi.

SUMMARY

It has proved possible to transform an extreme rough variant of pneumococcus back to the rough state by the action of the desoxyribonucleate fractions of either rough or Type III smooth pneumococci. In a second step, the rough pneumococci produced by this transformation were further changed to the Type III smooth state by means of the desoxyribonucleate fraction of Type III smooth organisms. With use of the Type III desoxyribonucleate, the two steps could be accomplished successively, but not simultaneously, during the growth of a single inoculum of the extreme rough form.

These findings have been interpreted as indicating that the desoxyribonucleic acid fraction of Type III smooth pneumococci contains two transforming principles of differing specificity, while the same fraction of rough pneumococci contains but one of these principles.

Two distinct spontaneous variants of Type III smooth pneumococci have been isolated which seem to differ from normal Type III smooth pneumococci in synthesizing smaller amounts of the specific polysaccharide. Tests have indicated that these variant Type III races differ from the normal in possessing altered Type III transforming principles. Each of the new transforming agents when influencing rough bacteria, is strictly specific in its action, inducing as it does the formation of the corresponding variant Type III pneumococci.

Interaction between the two new transforming principles and rough pneumococci can lead to the production of normal Type III organisms, although neither principle alone can do it. This is interpreted as indicating that the two mutated Type III transforming principles are qualitatively different from each other.

Another kind of two-step transformation was accomplished by converting rough pneumococci first into the variant Type III pneumococci which produced very little specific polysaccharide, and then by transforming these latter into normal Type III organisms.

After the two-step transformation of the extreme rough pneumococcus, both transforming principles used to effect this can be recognized in the Type III smooth pneumococci finally recovered. By contrast, in the two-step transformation of the rough pneumococcus by way of an intermediate smooth form, only the second transforming principle can be obtained from the resulting fully smooth organisms.

The meaning of these facts is discussed.

BIBLIOGRAPHY

1. Luria, S. E., *Bact. Rev.*, 1947, **11,** 1.
2. Avery, O. T., MacLeod, C. M., and McCarty, M., *J. Exp. Med.*, 1944, **79,** 137.
3. McCarty, M., and Avery, O. T., *J. Exp. Med.*, 1946, **83,** 89.
4. McCarty, M., and Avery, O. T., *J. Exp. Med.*, 1946, **83,** 97.
5. MacLeod, C. M., and Krauss, M., *J. Exp. Med.*, 1947, **86,** 439.
6. McCarty, M., Taylor, H. E., and Avery, O. T., in Cold Spring Harbor Symposia on Quantitative Biology, Cold Spring Harbor, Long Island Biological Association, 1946, **11,** 177.
7. Dubos, R. J., and Avery, O. T., *J. Exp. Med.*, 1931, **54,** 51.
8. Griffith, F., *J. Hyg.*, Cambridge, Eng., 1928, **27,** 113.
9. Blake, F. G., and Trask, J. D., *J. Bact.*, 1933, **25,** 289.

EXPLANATION OF PLATE 15

Photographed from sectors of the same blood-agar plate. × 40.

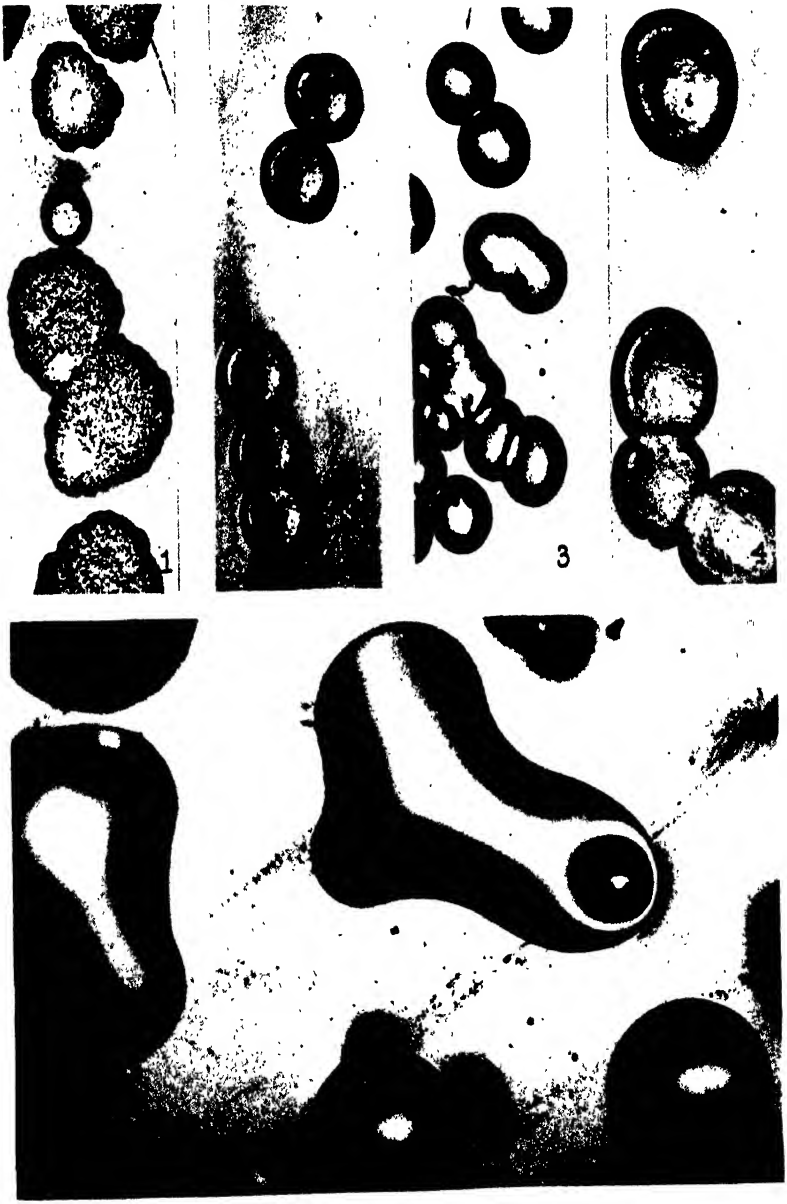
FIG. 1. Colonies of the extreme rough strain, ER.

FIG. 2. Colonies of the rough strain R36A.

FIG. 3. Colonies of the intermediate smooth strain, SIII-1.

FIG. 4. Colonies of the intermediate smooth strain, SIII-2.

FIG. 5. Colonies of the SIII-N strain, A66.



(Taylor: Transforming agents from pneumococcus variants)

[Reprinted from the PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE, March, 1949, Vol. 70, No. 3, pp. 547–551]

MULTIPLICATION OF INFLUENZA VIRUS IN DEAD CHICK EMBRYOS

BY O. LAHELLE AND FRANK L. HORSFALL, JR.

(From the Hospital of The Rockefeller Institute for Medical Research)

Recently, the cultivation of *Rickettsia prowazeki* in dead chick embryos was described by Rabinowitz, *et al.*[1] These workers showed that the rickettsiae employed multiplied abundantly in embryos which, although dead, still contained surviving cells. They confirmed the earlier work of Bucciante[2] and demonstrated that dead embryos may contain living cells for surprisingly long periods. After holding 3-day-old embryos at 4°C for 24 hours, then at room temperature for 7 days, and finally at 37°C for 16 days, they were able to culture living cells from the embryo or its membrane in plasma clots.

It seemed of both practical and theoretical interest to determine whether influenza virus would multiply in dead chick embryos. A number of experiments were carried out in which the time and temperature, at which embryos were held, were varied over wide ranges. The results obtained indicate that the PR8 strain of influenza A virus is capable of multiplication in the tissues of dead chick embryos if incubation at 35°C is sufficiently prolonged. They show also that the virus multiplies in dead embryos to an extent comparable to its multiplication in the living embryo.

Materials and Methods. The PR8 strain of influenza A virus was employed. It was cultivated in the allantoic sac of 9-day embryos which were incubated at 39°C before inoculation and at 35°C thereafter. Infected allantoic fluid gave hemagglutination and embryo infectivity titers of the order of 1:2000 and $10^{-8.5}$, respectively. Infected fluid was diluted in sterile nutrient broth; 0.2 cc of either a 10^{-3} or 10^{-6} dilution was employed as the inoculum.

The embryos of White Leghorn eggs were used. They were incubated at 39°C for periods ranging from 5–10 days. In certain experiments, after inoculation of the virus into the allantoic sac, embryos were held at room temperature for 3–10 days, and thereafter incubated at 35°C for 2–10 days. In other experiments, normal embryos were chilled at 4°C for either 20 or 96 hours, some of the former then held at room temperature for 3 days, after which they were inoculated into the allantoic sac with virus. Following this, the embryos were held at room temperature for an additional period of 2–10 days, and finally incubated at 35°C for 2–10 days. Groups of 2–3 embryos were employed and a variety of different experimental conditions were used. Specimens of allantoic

[1] Rabinowitz, E., Aschner, M., and Grossowicz, N., PROC. SOC. EXP. BIOL. AND MED., 1948, 67, 469.

[2] Bucciante, L., *Arch. exp. Zellforsch.*, 1931, 11, 397.

fluid were removed repeatedly from individual embryos, usually at intervals of 2 days. In this way it was possible to follow the multiplication of the virus in individual embryos.

Measurement of the concentration of virus in the extra-embryonic fluid was carried out by the hemagglutination technique as described previously.[3] Washed chicken RBC in a final concentration of 0.25% were used. The allantoic fluid dilution-RBC mixtures were held at room temperature for one hour. The titration end point was taken as that dilution which gave a 2+ reaction. Hemagglutination-inhibition tests[3] with specific immune rabbit sera were carried out with a constant dilution of serum capable of inhibiting hemagglutination by 128 units of the homologous virus.

Virus infectivity titrations were carried out as described previously.[4] Serial tenfold dilutions of fluid were inoculated into the allantoic sac of 9-day chick embryos and, after incubation at 35°C for 48 hours, the allantoic fluids were removed and tested by the hemagglutination procedure. The infectivity end point was calculated in the usual way.

Results. It was found that the time required to kill embryos at room temperature varied somewhat with their age. Thus, 5-day embryos held at room temperature for one day were almost always dead; 8-day embryos held for 2 days were usually dead, and invariably when held for 4 days; 10-day embryos held for 3 days were usually dead, and invariably when held for 4 days. Only 5-day embryos were held at 4°C and in every instance it was found that 20 hours at this temperature resulted in death. Embryos were considered to be dead when spontaneous movements had ceased, blood vessels showed no pulsation and no further development occurred.

The results obtained with embryos held for long periods at room temperature after inoculation with the PR8 strain are shown in Table I. It was found that multiplication of the virus did not occur when embryos were kept at room temperature for periods up to 8 days and not incubated at 35°C thereafter. However, with inoculated embryos which had been held at room temperature for 7–10 days, and then incubated at 35°C for periods ranging from 2–10 days, definite evidence of multiplication of the virus was obtained. In contrast to what occurs in the allantoic sac of living embryos,[5] maximum hemagglutination titers were not obtained with dead embryos until the period of incubation at 35°C had been extended to 6 or 8 days. In certain instances hemagglutination titers as high as 1:2000 and embryo infectivity titers of at least 10^{-5} were obtained with fluid from dead embryos. Hemagglutination-inhibition tests with immune sera served to identify the virus; complete inhibition was demonstrated repeatedly with anti-PR8 serum, while anti-Lee serum did not inhibit

[3] Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49.
[4] Hirst, G. K., *J. Immunol.*, 1942, **45**, 285.
[5] Pearson, H. E., *J. Bact.*, 1944, **48**, 369.

the reaction. Even when the inoculum was as small as 10^{-6}, *i.e.* approximately 10^2 E.I.D., multiplication of the virus occurred. It is noteworthy that inoculated embryos which were held for 10 days at room temperature were capable of supporting the multiplication of influenza virus when they were incubated at

TABLE I

Multiplication of PR8 in the Allantoic Sac of Chick Embryos Held at Room Temperature for Long Periods After Inoculation

Age of embryos (days)	Inoculum 0.2 cc intra-allantoic (dilution)	After inoculation		No. embryos	Allantoic fluid	
		Room temp. 22–25°C. (days)	35°C (days)		Hemagglut. titer*	Embryo-infectivity titer
5	10^{-3}	3–8	—	3	0,0,0	
7	"	"	—	"	0,0,0	
9	"	"	—	"	0,0,0	
5	"	7	2	2	8,16	
"	"	"	4	"	128,128	
"	"	"	8	"	256,256	$>10^{-5}$
8	"	"	2	"	0,0	
"	"	"	4	"	0,128	
"	"	"	8	"	512,2048	$>10^{-5}$
10	"	"	2	"	0,0	
"	"	"	4	"	0,0	
"	"	"	8	"	0,32	
5	"	10	3	"	0,32	
"	"	"	5	"	8,256	
"	"	"	7	"	512,2048	$>10^{-5}$
8	"	"	3	"	0,0	
"	"	"	5	"	0,32	
"	"	"	7	"	C,128	$>10^{-5}$
5	10^{-6}	7	2	3	0,0,128	
"	"	"	6	"	C,1024,1024	
"	"	"	10	"	C,2048,2048	
5	"	10	3–10	"	0,0,0	

* Hemagglutination titer of allantoic fluid from individual embryos.
C = Bacterial contamination.

35°C. It should be reiterated that, irrespective of the age of the embryos employed, none was capable of surviving for longer than 4 days at room temperature.

The results obtained with embryos which were killed by chilling at 4°C, and held for various periods at room temperature before inoculation with the PR8 strain, are shown in Table II. In these experiments all embryos employed were 5 days of age. In the large majority of instances embryos which had been inoculated after death were capable of supporting the multiplication of influenza virus if they were incubated at 35°C. Moreover, dead embryos held at room

temperature for 3 days before inoculation or 4 days thereafter supported viral multiplication. Maximum hemagglutination titers comparable to those obtained with live embryos were demonstrated with dead embryos when incubation at 35°C was carried out for 4–6 days. Quantities as small as 10^3 E.I.D.

TABLE II

Multiplication of PR8 in the Allantoic Sac of Chick Embryos Held at 4°C or at Room Temperature for Long Periods Before Inoculation

Before inoculation		Inoculum 0.2 cc intra-allantoic (dilution)	After inoculation		No. embryos*	Allantoic fluid hemagglut. titer†
4°C (hr)	Room temp. 22–25°C (days)		Room temp. 22–25°C (days)	35°C (days)		
20	—	10^{-3}	4	2	3	64,64,64
"	—	"	"	6	"	512,256,256
"	—	"	"	10	"	128,128,64
"	—	"	9	2–6	"	0,0,0
"	—	10^{-6}	4	2	"	0,0,0
"	—	"	"	4	"	C,2048,64
"	—	"	"	10	"	C,2048,2048
"	—	"	7	3–9	"	C,0,C
"	—	"	10	2–5	"	C,0,C
—	4	10^{-3}	4	3	"	256,0,64
—	"	"	"	6	"	32,1024,128
—	"	"	8	3	2	64,0
—	"	"	"	7	"	16,C
20	3	"	4	3	3	64,0,0
"	"	"	"	6	"	512,C,0
"	"	"	"	10	"	512,C,C
"	"	"	8	3–7	"	0,0,0
96	—	"	—	2	"	0,0,8
"	—	"	—	5	"	512,512,1024
"	—	"	—	11	"	2048,2048,2048
"	—	"	2	3–9	"	0,0,0
"	—	"	4	3–10	"	0,C,C
"	—	"	8	3–7	"	0,0,0

* All embryos were 5 days of age when inoculated.
† Hemagglutination titer of allantoic fluid from individual embryos.
C = Bacterial contamination.

of virus were capable of initiating multiplication in such embryos. It is of interest that even when embryos were held at 4°C for as long as 96 hours, they were still capable of supporting multiplication of the virus. It should be emphasized that such embryos had been dead for at least 3 days before inoculation.

In other experiments embryos were frozen at −30°C and held for 20 hours. They were then thawed, inoculated with a 10^{-3} dilution of virus and incubated at 35°C for prolonged periods. Under these conditions no evidence of multiplication was obtained in any instance.

The extra-embryonic fluid obtained from dead embryos had a gross appearance very different from that which is withdrawn from live embryos. In most instances the fluid was cloudy or turbid and frequently it contained some yolk, albumin, or both. As might be expected, hemagglutination reactions obtained with such fluids were somewhat abnormal, especially in the lower dilutions. However, in the higher dilutions hemagglutination appeared to be nearly normal and the end point was usually determined easily.

Discussion. Multiplication of influenza virus in the tissues of dead chick embryos is probably analogous to its multiplication in chick embryo tissue culture medium. It was surprising, however, to find that when dead embryos were incubated at 35°C for a sufficient period the virus titer became much higher than is found in liquid tissue culture.[6] The evidence obtained does not suggest that the virus is capable of multiplying in the absence of living cells. It has been demonstrated clearly[1,2] that in embryos killed by chilling at 4°C or by prolonged storage at room temperature, living cells are present. Presumably these cells carry on their usual metabolic functions when held at 35°C. It seems probable that the long period of incubation necessary for the development of maximal virus titers may be related to and dependent upon the multiplication of such living cells in both the dead embryo and its membranes. In this connection it will be recalled that when embryos were frozen at −30°C they were thereafter incapable of supporting viral multiplication.

It is apparent that these results may be of practical usefulness, especially in field work where attempts are made to recover viruses from infected persons or animals. In the case of influenza virus, at least, it seems evident that it is not necessary to observe special precautions with chick embryos. When adequate laboratory facilities are lacking, dead embryos which have been stored for considerable periods may prove satisfactory for the recovery of the infectious agent.

Summary. Chick embryos killed by prolonged storage at room temperature or 4°C are capable of supporting the multiplication of influenza virus upon prolonged incubation at 35°C.

[6] Weller, T. H., and Enders, J. F., Proc. Soc. Exp. Biol. and Med., 1948, **69**, 124.

[Reprinted from the ARCHIVES OF INTERNAL MEDICINE, September, 1948, Vol. 82, No. 3, pp. 229–250]

BACTERIOLOGIC AND IMMUNOLOGIC STUDIES ON PATIENTS WITH HEMOLYTIC STREPTOCOCCIC INFECTIONS AS RELATED TO RHEUMATIC FEVER

BY SIDNEY ROTHBARD, M.D., ROBERT F. WATSON, M.D., HOMER F. SWIFT, M.D., AND ARMINE T. WILSON, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research*)

Numerous investigators have studied the antibody responses of patients with either complicated or uncomplicated streptococcic infections, but only a few workers[1] have dealt with more than one or two of the antibodies to the known antigenic components of group A hemolytic streptococci. Moreover, reports which have dealt with the antibody response of patients with streptococcic complications and sequelae rarely included the period of the acute streptococcic infection in the study.

Antistreptolysin O and antifibrinolysin have been the two antibodies most commonly studied. Todd[2] originally showed that the serum antistreptolysin O titer was increased after hemolytic streptococcic infections and during the active stage of rheumatic fever. Tillett, Edwards and Garner[3] were the first to show that the plasma clot of most patients convalescent from acute hemolytic streptococcic infections became resistant to lysis by a standard culture of hemolytic streptococci, and Hadfield, Magee and Perry[4] observed that the plasma clots from patients with rheumatic fever were also resistant to lysis in the great majority of cases. These observations have been confirmed by many other workers. Antibodies in convalescent human serums against several other antigens of group A streptococci have also been studied, namely, anti-streptolysin S,[5] type-specific anti-M precipitins,[6] group A specific anti-C carbohydrate precipitins,[1a] and bactericidal and bacteriostatic antibodies.[7] Type-specific agglutinins for homologous streptococci were also observed,[8] and mouse-protective antibodies were demonstrated in serums of patients recovering from group A streptococcic infections.[9]

Not only have many of these serologic studies revealed a close relationship between hemolytic streptococcic infections and rheumatic fever but they have also been applied by certain investigators to show possible differences in the antibody response between streptococci-infected patients in whom rheumatic fever developed and those in whom it did not. For instance, Todd, Coburn and Hill[5] observed a contrast between the low (but increased above the normal) antistreptolysin S titer and the high antistreptolysin O titer in the serums of patients with active rheumatic fever, whereas both antistreptolysin O and antistreptolysin S titers were definitely increased in the absence of rheumatic activity after streptococcic infections. Swift and Hodge[6a] observed that anti-M

precipitins appeared later in patients in whom rheumatic fever developed than in those in whom this sequela failed to appear. Coburn[6b] found that the antibody responses to streptolysin O and the type-specific M protein were delayed in rheumatic patients in whom recurrent attacks of rheumatic fever developed as compared with those who escaped recurrences after hemolytic streptococcic pharyngitis. Kuttner and Krumwiede,[10] on the other hand, have not been able to find a delayed antibody response to streptolysin O in patients in whom rheumatic fever developed after hemolytic streptococcic infections.

It is now generally believed that rheumatic fever is usually, if not invariably, preceded by infections with group A hemolytic streptococci. The mechanism by which the streptococcic infection activates rheumatic fever is still unknown. If by means of antibody studies it could be shown that rheumatic fever following streptococcic infections is characterized by a different antibody pattern than that which occurs in comparable subjects in whom rheumatic fever does not develop, then further insight might be gained concerning the mechanism by which streptococcic infections activate this disease. It appeared advisable, therefore, to extend this type of investigation for the following reasons: Many previous immunologic studies began with the period of rheumatic fever and neglected the acute streptococcic phase. Most previous investigators employed only one or two antibody reactions to hemolytic streptococcic antigens. There is still disagreement as to whether the antibody response of the rheumatic patient differs appreciably from that of the non-rheumatic patient.

All patients were followed from the time of onset of the acute streptococcic infection through the period of complications and sequelae until convalescence was well established. Serums obtained at weekly intervals were tested for the following responses:

1. Reactions induced by group A streptococci without regard to type, which included (*a*) antistreptolysin O, (*b*) antifibrinolysin, (*c*) precipitins against group A specific carbohydrate C and (*d*) precipitins against a nucleoprotein fraction obtained from group A streptococci.

2. Reactions to type-specific components of group A streptococci, which included (*a*) type-specific anti-M precipitins and (*b*) type-specific bacteriostatic antibodies.

3. Reactions having no demonstrable specific relationship to streptococcic infections, which included (*a*) precipitins against pneumococcic "C" polysaccharide and (*b*) phase precipitin reactions (in 9 patients in whom rheumatic fever developed).

Materials and Methods

Clinical Study.—From October 1940 to June 1944 a series of rheumatic and non-rheumatic subjects suffering from definite group A streptococcic nasopharyngitis was observed. They were invariably hospitalized early in the course of the primary strepto-

coccic disease. To prevent cross infections with other types of streptococci, each patient was isolated until two successive cultures of the throat and nasopharynx, taken at least twenty-four hours apart, revealed no hemolytic streptococci. On their admission to the hospital, a complete history was recorded and a physical examination made; daily changes in signs and symptoms were uniformly documented. Routine total erythrocyte, leukocyte and differential counts, estimations of hemoglobin content, and erythrocyte sedimentation rate (Westergren), cultures of material from the nasopharynx and throat, examination of urine, electrocardiographic studies, and roentgenologic studies of the heart and lungs and the paranasal sinuses when indicated, were done at the time of entry to the hospital. Blood samples for serologic studies were obtained on admission, and at weekly intervals thereafter. In addition, serial weekly total leukocyte counts, examination of urine, electrocardiographic studies, determinations of erythrocyte sedimentation rates and cultures of material from the nasopharynx and throat (until two successive cultures taken twenty-four hours apart were sterile for hemolytic streptococci) were done.

Only symptomatic therapy was employed during the acute streptococcic phase of the illness, but patients with purulent complications were treated with sulfadiazine; many of them were also given therapeutic doses of sulfadiazine in an effort to clear up the streptococcic carrier state. All patients with rheumatic fever received adequate doses of salicylates to eliminate symptoms and fever, and this was continued throughout the period of rheumatic activity. For 1 patient it was necessary to use "pyramidon" (aminopyrine).

After discharge from the hospital, many of the rheumatic subjects were examined in the outpatient department at intervals of three to four weeks; if subsequent infections occurred, they were immediately readmitted for study.

Bacteriologic Methods.—Cultures of the nasopharynxes were obtained by passing a sterile swab through each nostril to the posterior pharyngeal wall; throat cultures were made by swabbing both tonsils or tonsillar fossae and the posterior pharynx. The swabs were streaked immediately in duplicate on fresh 5 per cent rabbit and sheep blood agar plates. Typical representative colonies were then picked and streaked on other blood agar plates for further identification or transferred directly to broth for classification. The hemolytic streptococci were grouped and typed by the precipitin technic.[11]

Serologic Technics.—Determinations of antistreptolysin O titers were made according to Todd's method[12] modified as previously described.[13] The antifibrinolysin tests were performed by the method of Tillett, Edwards and Garner[3] and that of Boisvert.[14]

For the various precipitin tests, the following reagents were employed. The group A specific carbohydrate C was made by Fuller's formamide method[15] and used in the dilution which gave maximal precipitation with hyperimmune rabbit serum. The type-specific M extracts, prepared according to Lancefield's technic,[11] were tested for type specificity with absorbed immune rabbit serum of homologous and heterologous types and were proved free of group-specific C substance by the employment of suitable antiserums. The streptococcic nucleoprotein fraction[16] was used in a concentration of 5 mg. of dry weight per cubic centimeter of diluent. The C-reactive protein was determined according to the method of Tillett and Francis[17] with a pneumococcus

C polysaccharide[18] in a concentration of 10 mg. per hundred cubic centimeters. The phase precipitin test[19] was performed according to the method of Coburn and Pauli.[20]

The reaction to the bacteriostatic test, previously described,[7d] was considered positive only if there was at least a 2 plus difference in growth from at least two different dilutions as compared with the corresponding streptococcus dilution control.[21]

OBSERVATIONS AND RESULTS

Clinical and Bacteriologic Observations.—A total of 153 patients were studied, 128 males and 25 females. Their ages ranged between 3 and 45 years; 19 patients were in the first decade, 54 in the second, 62 in the third, 14 in the fourth and 4 in the fifth. The 153 patients suffered a total of 169 definite hemolytic streptococcic infections of the upper respiratory tract, and in every instance relatively large numbers of hemolytic streptococci, often in almost pure culture, were recovered from the patients' nasopharynxes. Fifty-four different infections were experienced by a group of 39 patients known to have had one or more attacks of rheumatic fever previously; 115 infections occurred among 114 nonrheumatic subjects. In several cases definite clinical evidence of infection was not present, although large numbers of hemolytic streptococci appeared in nose and throat cultures of patients who shortly before had not harbored these micro-organisms and who later had a definite increase of antistreptolysin O. Among the 39 known rheumatic subjects who suffered 54 hemolytic streptococcic infections, there were 17 recurrences of rheumatic fever. Three of these patients each had two separate attacks of rheumatic fever after infections with different types of group A streptococci. Rheumatic fever developed in 21 of the 114 previously nonrheumatic subjects who experienced 115 streptococcic infections. The patients with rheumatic fever had polyarthritis and carditis and at the same time increased leukocyte counts and erythrocyte sedimentation rates; a few patients,[22] however, with accompanying secondary rises in blood leukocyte counts and/or sedimentation rates had only carditis after their streptococcic infections.

Scarlet fever characterized 114 of the 169 infections, but in the following analysis the group with this disease are not differentiated from patients with tonsillitis or pharyngitis without a rash, since many of the patients who in the same epidemic failed to show a rash were infected with the same type of streptococcus as those in whom a scarlatiniform rash developed.

The data given in table 1 show the distribution of the serologic groups and types of streptococci cultured in material from the 153 patients during their 169 attacks of acute pharyngitis; 102 of the attacks were uncomplicated, 29 resulted in purulent complications during the first to the fourth week (average 1.6 weeks) due to the same streptococcic type which caused the initial infection and 38 were followed by acute rheumatic fever. In 4 of these 38 there were also purulent complications. Rheumatic sequelae occurred during the first

to the eighth week (average 3.4 weeks) after the onset of the streptococcic infection.

Six of the streptococcic strains belonged to group C, one to group G and one hundred and sixty-two to group A. Twenty-two, or 13.6 per cent, of the group A strains failed to type by the anti-M precipitin method with diagnostic serums representing thirty-seven different serologic types, although three of these were proved to contain the T antigen of type 14 by the agglutination technic. In 5 instances two types were recovered; the type listed first in table 1 was considered probably responsible for the infection. Type 19 predominated in all three clinical groups and made up 45.3 per cent of the infecting strains of group A streptococci. The preponderance of this type among the patients was due to the inclusion of naval personnel involved in an epidemic of scarlet fever previously reported.[23] It is of interest to note, however, that nine other serologic types, as well as seven unclassified strains, caused infections that were followed by rheumatic fever.

It is also of interest that the 7 hemolytic streptococcic infections not of group A were not followed by rheumatic fever although 5 of them occurred in patients who were known to have had rheumatic fever previously and who were therefore presumably susceptible to the disease. In fact, in 1 of these rheumatic subjects a recurrent attack of rheumatic fever subsequently developed after a group A streptococcic infection. It is significant that no single serologic type of group A streptococci could be correlated with the development of either purulent complications or rheumatic fever. The distribution of the various serologic types was approximately the same in patients who made uneventful recoveries as in those in whom complications or sequelae developed.

Antibody Studies.—A summary of the results of the antistreptolysin O titrations and the antifibrinolysin determinations made on the serums and plasma respectively of the patients without complications and of those who suffered purulent complications or rheumatic fever after streptococcic pharyngitis is presented in table 2. The data cover all the determinations made at the time of the acute streptococcic infection and weekly thereafter until the patients were discharged from the hospital. In some instances, further determinations were made from blood samples obtained on subsequent visits to the clinic at three to four week intervals.

A significant increase in the serum antistreptolysin O titer was considered to have occurred only if it increased by two or more dilutions over that shown at the time of the initial determination. In accordance with this criterion, the serum antistreptolysin O titer increased in 76.4 per cent of the patients with uncomplicated pharyngitis, in 75.9 per cent of those in whom purulent complications developed and in 84.2 per cent of those in whom rheumatic fever developed (table 2). The interval between the onset of infection and the beginning of the rise in the antistreptolysin O titer was essentially the same for the

three groups, namely, 2.1, 2.2 and 2.0 weeks respectively. The average interval between the onset of infection and the time at which the maximal titer was

TABLE 1

*Serologic Classification of Streptococci Isolated from Patients with Acute Pharyngitis Correlated with the Development of Complications**

		No. of Streptococcic Infections Associated with			Total
		No Complications	Purulent Complications	Rheumatic Fever	
Group A	Type				
	1	3	1	1	5
	3	9	1	1	11
	3–14†	1			1
	5	1			1
	6	5	2	2	9
	6–14†		1		1
	12	2			2
	14	3		2	5
	17	3	2	2	7
	18	2			2
	19	38	13	17	68
	19–6†	1	1	1	3
	23	1	1		2
	26	2	1	3	6
	29	1			1
	30	6	2		8
	32			1	1
	33	1		1	2
	38	3			3
	39	1			1
	45	1			1
	NC‡	11	4	7	22
Group C		6			6
Group G		1			1
Totals		102	29	38	169

* A total of 153 patients suffered 169 different streptococcic infections.

† Streptococci of two types were recovered from these patients. The type listed first was considered probably responsible for the infection.

‡ These strains could not be classified into types by the precipitin technic, but one in the group with no complications and two in the group with purulent complications were proved by the agglutination method to contain the T antigen of type 14.

reached was 4.7 weeks for the patients with no complications, 3.9 weeks for the patients in whom purulent complications developed and 5.5 weeks for those in whom rheumatic fever developed. In table 2 the magnitude of the serum

antistreptolysin O response is expressed as the ratio between the maximal and the initial titers. When the responses of these three groups of patients are compared on this basis, it is evident that the average rise was 9.3 times for the group of patients in whom purulent complications developed, 5.1 times for those in whom rheumatic fever developed and only 3.8 times for those who had

TABLE 2

Summary of Weekly Determinations of Antistreptolysin O and Antifibrinolysin Correlated with the Type of Clinical Reaction to Streptococcic Infections

Complications and Sequelae		None	Purulent	Rheumatic Fever	Total
Antistreptolysin O					
Number of infections		102	29	38	169
Increased titers:	Number	78	22	32	132
	Per cent	76.4	75.9	84.2	78.1
Beginning of rise*	Weeks	2.1	2.2	2.0	2.2
Maximal level*	Weeks	4.7	3.9	5.5	4.8
Maximal level (units/cc.)†: Initial level (units/cc.)	Ratio	3.8	9.3	5.1	5.0
Antifibrinolysin					
Number of infections		85	26	24	135‡
Increased titers:	Number	46	17	19	82
	Per cent	54.1	65.4	79.2	60.1
Beginning of rise*	Weeks	2.5	2.7	2.4	2.5
Maximal level*	Weeks	3.4	3.2	3.0	3.2

* Based on average time in weeks after onset of infection.

† An expression of the magnitude of the response, based on the average of the ratios $\frac{\text{maximal level}}{\text{initial level}}$ calculated for each individual patient.

‡ In an additional 34 infections (20 per cent) antifibrinolysin determinations could not be made because of high or maximal resistance of the plasma clot to fibrinolysis at the onset of infection.

no complications. The data seem to indicate that, on the average, patients in whom rheumatic fever develops are more likely to have significant serum antistreptolysin O responses, with slightly delayed maximal titer, and are also likely to have a more intense response than the patients with uncomplicated disease but a less intense response than those who suffer purulent complications. These results, however, are of doubtful significance since the groups studied are small. Moreover, when the individual patients are considered, it is found that there is great variation and considerable overlapping among the members of the three groups.

A total of 132 (78 per cent) of the 169 streptococcic infections resulted in significant rises in the antistreptolysin O titer. The average time for the beginning of the rise in titer was 2.2 weeks; for the maximal level to be reached, 4.8 weeks were required. The average maximal-initial ratio was 5.0 times.

The antifibrinolysin was considered significantly increased only if between admission and discharge there was an increase of 2 plus in the results. These tests were less satisfactory for comparison than were the determinations of antistreptolysin O because this test, as employed here, was not quantitative[24] and in many instances increase in antifibrinolysin could not be measured because the initial level was too high. Even though the numbers are small, it is noteworthy (table 2) that 79.2 per cent of the patients in whom rheumatic fever developed showed a significant increase in their antifibrinolysin titers whereas only 54.1 per cent of the group without complications and only 65.4 per cent of the patients in whom purulent complications developed showed increased titers. The average time of onset of the initial increase in resistance to lysis of the plasma clot and the time when the maximal level was reached were essentially the same for each group.

In a total of 83 (60 per cent) of 135 infections significant increases in the antifibrinolysin titers were observed. The beginning of the rise of antifibrinolysin and the maximal level occurred in 2.5 and 3.2 weeks respectively. Unfortunately, it was not possible to determine the duration of the increase of either antistreptolysin O or antifibrinolysin because many patients were discharged from the hospital before the antibody responses returned to the initial level.

Additional Antibody Studies.—From the entire group of 153 patients, 71 who suffered 83 different group A streptococcic infections were selected for a more comprehensive study of their antibody responses. These patients were divided into three groups, on the basis of their clinical response to the streptococcic infections, in the same manner in which the large group was considered, namely, (1) those in whom complications failed to develop, (2) those in whom purulent complications developed and (3) those in whom rheumatic fever developed. In addition to determinations of antistreptolysin O and antifibrinolysin, the serums of these patients were tested at weekly intervals for the presence of streptococcic bacteriostatic antibodies and precipitins directed toward the type-specific M protein, the group-specific C carbohydrate and a nucleoprotein fraction of group A streptococci and for the presence of C-reactive protein (against pneumococcus C).

In table 3 are summarized the findings with respect to four different streptococcic antibodies which developed in these 71 patients. The results of the antistreptolysin O titrations for these three groups are essentially the same as those found for the three larger groups and recorded in table 2. The percentage of patients who showed increased antifibrinolysin titers is somewhat higher than that in the larger group. This slight difference is probably due to the

difference in the number of cases summarized in the two tables. Because larger groups tend to be more representative, we believe that the figures given in table 2 reflect more accurately the comparative differences between the three groups of patients than those in table 3.

The bacteriostatic and anti-M precipitin tests were employed to determine the presence of type-specific antibodies in the serums of these patients; the results are recorded in table 3. Eighty-eight per cent of the patients in whom rheumatic fever developed had demonstrable type-specific bacteriostatic antibodies, whereas 69 per cent of those with purulent complications and 67 per cent of those without any complications had them. In general, in the patients in whom rheumatic fever developed there was also a slight delay in the appearance of the bacteriostatic antibodies as compared with the two other groups of patients; the individual values, however, spread widely about the average, and some patients (table 3) in whom rheumatic fever developed showed a bacteriostatic antibody response as early as did those in whom rheumatic fever failed to develop after their streptococcic infection. In general the period for which these antibodies lasted could not be determined because they were still present when the patients left the hospital. In most instances, however, they persisted for at least many months and in some for over two years.

Although the results of the bacteriostatic test were strikingly type specific, the precipitin reactions with homologous and heterologous M extracts showed little evidence of type specificity. The highest incidence (85.3 per cent) of positive reactions, however, was again found in patients in whom rheumatic fever developed, and the average time of appearance of these antibodies was delayed as compared with that in the other two groups. However, as shown in table 3, cross reactions with heterologous M extracts were so frequent that little reliance can be placed on the type specificity of the test. Among the patients tested, with a total of 83 infections, 53 showed reactions with homologous M extracts, but 39 of the 53, or 73.6 per cent, showed equally strong reactions with extracts of heterologous types. Furthermore, the serums which reacted with heterologous type extracts usually reacted as well with the homologous type extracts. The results probably can be interpreted best as evidence of response to non-type-specific streptococcic products.

The group-specific carbohydrate C antibody was found in only 5 of the 83 infections; four of the positive reactions occurred with serums of patients in whom attacks of rheumatic fever developed and one with the serum of a patient in whom a suppurative complication developed. The precipitin directed against the nucleoprotein fraction was detected in only 3 patients, in all of whom rheumatic fever developed. In these 3, antibodies against both group-specific C carbohydrate and the nucleoprotein fraction were observed.

The C-Reactive Protein.—This protein, contained in the alpha$_1$ globulin fraction,[25] is precipitated with dilute solutions of pneumococcus "C" polysac-

TABLE 3

Summary of Weekly Determinations of Different Streptococcic Antibodies Correlated with the Type of Clinical Reaction to Streptococcic Infections

Complications and Sequelae		None	Purulent	Rheumatic Fever*	Total
Antistreptolysin O					
Number of infections		33	16	34	83
Increased titers:	Number	23	12	29	64
	Per cent	69.7	75.0	85.3	77.1
Beginning of rise†	Weeks	2.4	2.3	2.0	2.4
Maximal level†	Weeks	4.8	3.8	5.8	5.3
Maximal level (units/cc.)‡ : Initial level (units/cc.)	Ratio	4.2	10.6	5.4	6.0
Antifibrinolysin					
Number of infections		27	15	21	63**
†Increased titers:	Number	17	12	17	46
	Per cent	62.9	80.0	80.9	73.0
Beginning of rise†	Weeks	3.1	2.5	2.3	2.6
Maximal level†	Weeks	4.2	3.2	3.0	3.5
Bacteriostatic Antibodies					
Number of infections		33	16	33	82
Increased titers:	Number	22	11	29	62
	Per cent	66.7	68.8	87.9	75.6
Beginning of rise†	Weeks	4.2	3.9	6.1	5.1
Range†	Weeks	2 to 10	2 to 8	1 to 13	
Anti-M Precipitins					
Number of infections		33	16	34	83
Increased titers with:					
(1) Homologous M antigen	Number	15	9	29	53
	Per cent	45.5	56.2	85.3	63.9
(2) Heterologous M antigens¶	Number	10	7	22	39
	Per cent	33.3	43.8	61.8	46.9
Beginning of rise†	Weeks	3.6	2.6	6.0	4.8
Range†	Weeks	1 to 8	1 to 5	1 to 23	

* Four of these patients also had purulent complications.

† Based on average time in weeks after onset of infection.

‡ An expression of the magnitude of the response; based on the average of the ratios $\frac{\text{maximal level}}{\text{initial level}}$ calculated for each individual patient.

** In an additional 20 infections, determinations of antifibrinolysin could not be made because of high or maximal resistance of the plasma clot to fibrinolysis at onset of infection.

¶ Reactions were usually observed with heterologous M antigens in serums which also gave reactions with homologous M antigens.

charide and is found in the serum during the acute phase of various infectious diseases.[26] Although C-reactive protein is apparently in no way related to the formation of antibodies, it seemed of interest to compare its occurrence and duration in the presence of complications and sequelae following group A streptococcic infections in rheumatic and nonrheumatic subjects.

The results shown in table 4 indicate that the C-reactive protein was observed in 63, or 75.9 per cent, of the 83 infections. In patients showing positive reactions, it was usually detected in the serums obtained during the acute streptococcic phase and during the time of purulent complication as well as in the period of greatest rheumatic activity. It occurred in 51.4 per cent of the patients without complications, in 75 per cent of those with purulent complications and in 97.6 per cent of those in whom rheumatic fever developed.[27] The presence of the C-reactive protein in the serums showed a close correlation with the intensity and duration of the inflammatory reaction of the host, as

TABLE 4

Summary of Weekly Determinations of the C-Reactive Protein in Patients with Different Types of Clinical Reactions to Streptococcic Infections

Complications and Sequelae		None	Purulent	Rheumatic Fever	Total
Number of infections		33	16	34	83
Positive reactions	Number	18	12	33	63
	Per cent	51.4	75.0	97.6	75.9

indicated by the elevated temperatures and increased serial erythrocyte sedimentation rates illustrated in charts 1, 2, 3 and 4. This protein persisted longest in the serums of patients with rheumatic fever.

Phase Precipitins.—Escherich and Schick,[28] Schlesinger[29] and Coburn and Pauli[30] described three different clinical phases in the development of rheumatic fever. Phase I represents the acute streptococcic infection, phase II an afebrile and often asymptomatic period and phase III the state of rheumatic fever. A precipitation reaction which sometimes occurs when serums taken in phase II and phase III are mixed has been described by Coburn and Pauli.[20] The serum in phase II was considered by them to contain an antigen and that taken in phase III an antibody. These authors recorded no precipitate obtained by mixing phase I and phase II serums or phase I and III serums.

Wedum and Wedum[31] observed phase precipitin reactions not only with serums from patients with rheumatic fever but also with those from patients with atypical pneumonia and nasopharyngitis, and occasionally with those from apparently normal blood donors.

In the present study, serums which were obtained from 9 patients in all

three phases in the evolution of their attacks of rheumatic fever were tested for phase precipitins. In certain cases faint rings were observed at the interface of the two serums, but this occurred as often between the serums obtained in phases I and II or phases I and III as it did between phase II and phase

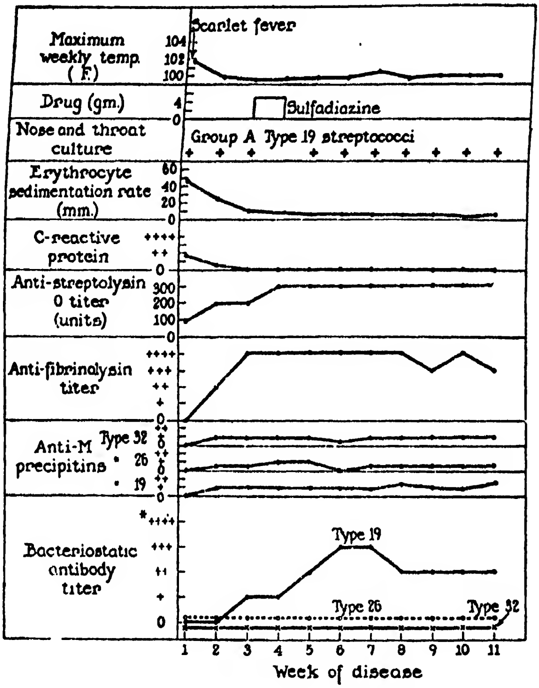


CHART 1. Course of a patient with an uncomplicated streptococcic infection of group A type 19.

* +, ++ and +++ represent significant bacteriostasis of streptococci in culture dilutions of 10^{-6}, 10^{-5} and 10^{-4} respectively; 10^{-6} usually represented 200 to 300 colonies per cubic centimeter.

III serums. In other cases no rings or precipitates were observed. It is not clear whether these faint rings represented true precipitin reactions.

Details of Findings in Typical Patients.—In charts 1, 2, 3 and 4 are shown the clinical course of 4 typical patients and the corresponding serologic and bacteriologic studies done. Chart 1 shows the course of a patient who made an uneventful recovery, chart 2 illustrates the observations made on a patient in whom a purulent complication developed, chart 3 illustrates the course of a patient in whom a primary attack of rheumatic fever developed after a streptococcic infection and chart 4 shows the observations made on a rheumatic subject with 3 different group A streptococcic infections of the upper respira-

tory tract. The first infection in the last patient was uncomplicated, but the second and third resulted in recurrent attacks of rheumatic fever. Certain

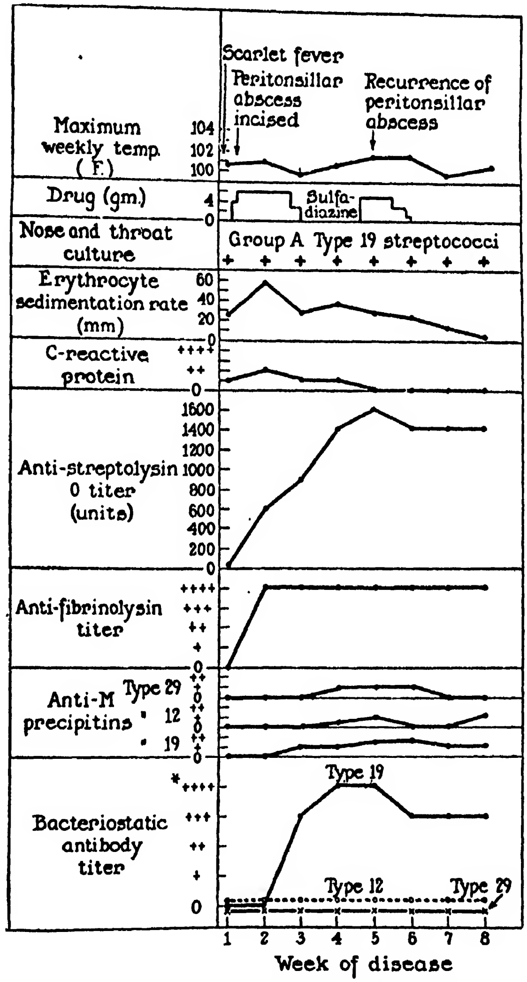


CHART 2. Course of a patient with a purulent complication following group A type 19 streptococcic infection.

* +, ++, +++ and ++++ represent significant bacteriostasis of streptococci in culture dilutions of 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} respectively; 10^{-6} usually represented 200 to 300 colonies per cubic centimeter.

points are noteworthy in all these charts: (1) the persistence of hemolytic streptococci in the nasopharynxes of the patients; (2) the close correlation of the increased erythrocyte sedimentation rates with the presence of the C-re-

active protein in the serum; (3) the cross reactions with the type-specific M protein extracts; (4) the type specificity of the bacteriostatic test and the long period for which the bacteriostatic antibodies were present, and (5) the special importance of the determinations of antistreptolysin O and the bacteriostatic

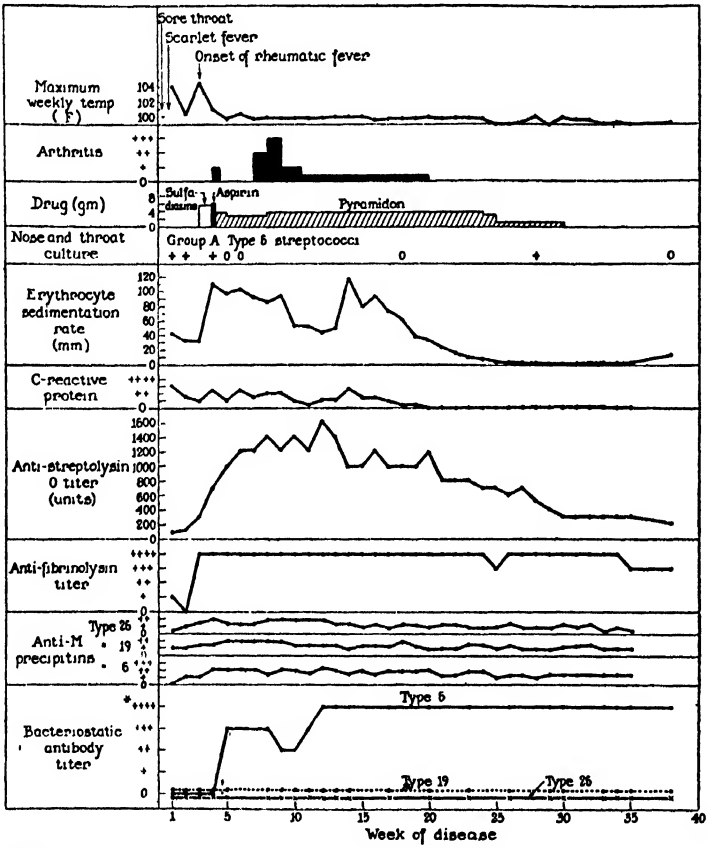


CHART 3. Course of a patient who had a primary attack of acute rheumatic fever after a group A type 6 streptococcic infection.

* +, ++, +++ and ++++ represent significant bacteriostasis of streptococci in culture dilutions of 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} respectively; 10^{-6} usually represented 200 to 300 colonies per cubic centimeter.

tests in patients with reinfection by different serologic types of group A streptococci.

Multiple Streptococcic Infections.—In 11 of the 153 patients more than one group A streptococcic infection developed. Ten were known rheumatic subjects, and 1 was a normal sibling of a brother with rheumatic disease. In 3 of

the patients 3 separate infections developed, and 2 developed in 8 others. In 10 of the patients different known types of hemolytic streptococci were cultured from the nasopharynxes in each infection, but in 1 patient with three separate attacks of disease of the upper respiratory tract the streptococci

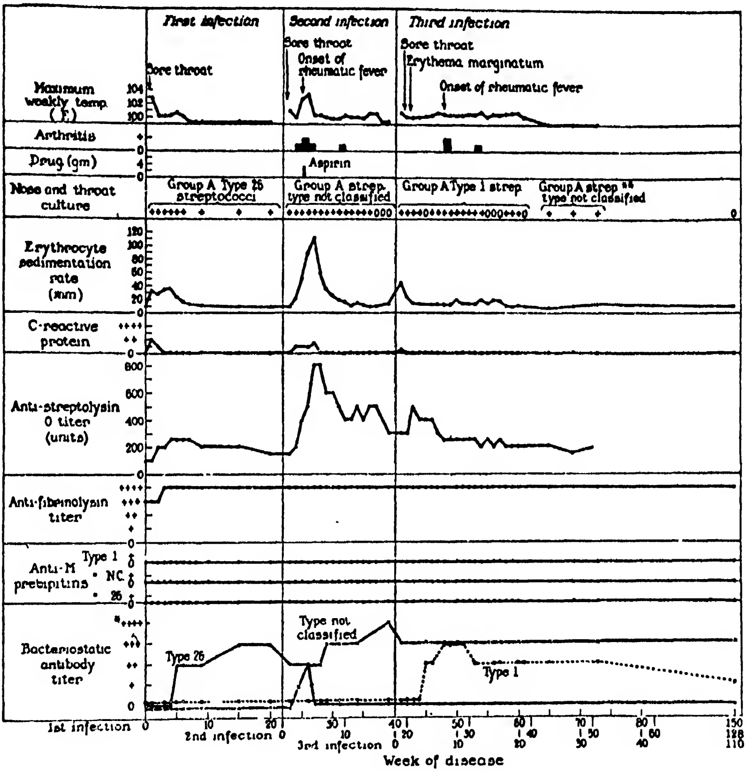


CHART 4. Course of a rheumatic subject with three different group A streptococcic infections.

* +, ++, +++ and ++++ represent significant bacteriostasis of streptococci in culture dilutions of 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} respectively; 10^{-6} usually represented 200 to 300 colonies per cubic centimeter.

** The streptococci of unclassified type comprised only a few colonies and were probably the same as those which caused the second infection.

could not be typed with the available serums by the precipitin technic although there were definite clinical findings and antibody responses in each of these infections. However, during 1 of the 3 infections, an indication was obtained that the strain was different from the others because bacteriostatic antibodies specific for this strain appeared in the patient's serum. The patients with multi-

ple but separate infections of the respiratory tract had a different type of streptococci with each infection.

Of primary interest also in this study is the fact that many of the rheumatic subjects suffered a number of nonstreptococcic infections such as rubella, herpes zoster, bacillary dysentery, appendicitis, acute epidemic conjunctivitis (pink eye), pneumococcic pneumonia and infections of the upper respiratory tract of unknown origin, but in no instance was rheumatic fever observed after these infections. In this respect our findings are in agreement with those of others.[32]

COMMENT

In order to determine whether there is any basic difference in the patterns of streptococcic antibodies in patients in whom rheumatic fever develops as compared with those who have purulent complications or make uneventful recoveries after hemolytic streptococcic infections of the upper respiratory tract, the immunologic responses to a variety of antigens were studied in fairly large groups of patients. It is well known[33] that different strains and serologic types of streptococci vary in their antigenic composition and thus they may evoke different responses in the host. Moreover, persons are also known to respond differently to the same antigen.

The antistreptolysin O test gives the most satisfactory index of previous streptococcic infections, probably because streptolysin O is an excellent antigen and the titer of this antibody can be accurately determined. The determinations of antifibrinolysin in this study were done before the quantitative method recently described by Kaplan[24] had been devised, and quantitative results were therefore not obtained. Because of its simplicity, it was hoped that the anti-M precipitin test could be used for determinations of type-specific antibodies. It was found, however, that frequent and marked cross reactions with heterologous M extracts made this test an unreliable index of type specificity in patients' serums. Similar difficulties were encountered by Thomas.[34] These reactions were not due to the group-specific C carbohydrate because this antigen had been eliminated from the M extracts. At present, the nature of these cross reactions is not understood, and the precipitin test as performed probably did not give a reflection of the type-specific antibodies in the patients' serums. The most reliable method for determining such antibodies was found to be the bacteriostatic test. In contrast to the anti-M precipitin test, no cross reactions were observed.

Since the antistreptolysin O reaction is the most sensitive test now available for detecting previous streptococcic infection, it was thought of interest to find out how often a rise in antistreptolysin O was accompanied with antifibrinolysin, bacteriostatic and anti-M precipitin responses. The data, obtained on 71 patients who had 83 group A streptococcic infections, are summarized

in table 5. Sixty-four of the 83 infections (77 per cent) induced a significant rise in the antistreptolysin O titer. During 14 of these 64, it was impossible to learn whether additional antifibrinolysin appeared because of the limitations of the method used; a rise in antifibrinolysin was observed during 40 of the remaining 50. In 45 of the 64 there was a rise in bacteriostatic antibodies. In 42 anti-M precipitins developed, but the significance of this finding is not clear, since in 32 of these cross reactions were observed. On the other side of the picture, in 16 of the 19 infections in which antistreptolysin O was not increased there was a rise in the bacteriostatic antibodies; in 1 of the remaining 3 there was an antifibrinolysin response, and in 2 anti-M precipitins developed. From

TABLE 5

Correlation of the Antistreptolysin O Response with Antifibrinolysin, Bacteriostatic and Anti-M Precipitin Reactions in Serums from Patients with 83 Group A Streptococcic Infections of the Upper Respiratory Tract

Antistreptolysin O	Positive 64			Negative 19		
	Positive	Negative	Not Determined	Positive	Negative	Not Determined
Antifibrinolysin	40	10	14	6	7	6
Bacteriostatic antibodies	45	19		16	2(1)*	
Anti-M precipitins	42 (32)†	22		11 (7)†	8	

* Figure in parentheses indicates that in one additional infection bacteriostatic antibodies could not be determined because streptococci isolated from the patient did not grow in whole blood of normal children even without addition of test serum.

† Figures in parentheses indicate the number in which reactions with heterologous M antigens were also present.

these results it can be seen that antibodies directed against one or several of the various antigenic components of group A streptococci appeared in the serums of all the patients after the infection. Moreover, in 50.5 per cent of the 83 infections three different antibodies, directed against streptolysin O, fibrinolysin and the type-specific M protein, appeared in the patients' convalescent serums.

Our findings are in accord with those of previous observers, who found that group A streptococcic infections of the upper respiratory tract precede the development of rheumatic fever. By means of immunologic studies, it is usually possible to establish the cause of the precursory pharyngitis in rheumatic patients as group A streptococci, although these micro-organisms may not be found on culture; the chances of obtaining this positive immunologic evidence are increased in proportion to the number of different antibody tests applied.

A comparison of the immune responses of the patients in whom rheumatic fever developed with those of the ones who escaped complications or sequelae

or in whom purulent complications developed shows that antistreptolysin O, antifibrinolysin, anti-M precipitins and type-specific bacteriostatic antibodies developed more frequently in rheumatic patients than in nonrheumatic ones. In the rheumatic group the maximal response of antistreptolysin O and the type-specific bacteriostatic antibody was delayed about two weeks as compared to that in the other two groups. When the average weekly bacteriostatic antibody responses of the rheumatic and nonrheumatic groups were reviewed, the former had an average lower response than the latter during each of the second to the fifth weeks. Whether these differences have significance with respect to the pathogenesis of rheumatic fever cannot at present be stated.

Mote and Jones[10] found that, compared to patients with streptococcic infections who recovered without sequelae, patients who had purulent complications as well as those in whom rheumatic fever developed exhibited a delay in the rise of antistreptolysin O. Coburn[6b] also found a delayed antistreptolysin O response in patients in whom rheumatic fever developed after streptococcic infections of the upper respiratory tract, and he stated that this is characteristic. Because the grouping of our cases was not the same as that of these authors, direct comparison is not possible. In our series the beginning of the rise of antistreptolysin O was not delayed. These results are similar to those obtained by Kuttner and Krumwiede.[10] In our patients the maximal response occurred later in the rheumatic group than in the nonrheumatic group. Since the difference was not great and the number of patients in each group was small and since there were great individual variations within each group, it cannot be concluded from this study that a delayed appearance of antistreptolysin O is characteristic of the rheumatic patient.

Swift and Hodge[6a] and Coburn[6b] also reported a delayed response of anti-M precipitins in patients in whom rheumatic fever develops. The precipitin reactions which we obtained with M extracts and patients' serums were similar to those reported by these authors; however, in view of the numerous cross reactions observed, the significance of this reaction is not clear. On the other hand, the delayed response of the bacteriostatic antibodies, which is type specific, may be significant. As previously indicated, this applies to groups and not to individuals.

One of three factors may be invoked to explain the difference between rheumatic and nonrheumatic groups of patients.

1. The groups are too small and the differences too slight to have significance.

2. The administration of salicylates to the rheumatic patients may have delayed formation of the type-specific antibodies.

3. The average statistical differences may indicate that it is part of the nature of rheumatic fever to have a delayed type-specific streptococcic antibody response to group A streptococcic infections.

Several previous investigations indicate that salicylates may depress the formation of antibodies.[35] In the present study, however, if salicylates had had this effect in the rheumatic group who had received them, it probably would have been reflected in the production of antistreptolysin O and antifibrinolysin as well as of anti-M precipitins and bacteriostatic antibodies (table 3). This was not the case, for the production of antibodies was greatest in the rheumatic group. The lag was evident only in the formation of type-specific antibodies. It should also be noted that salicylates were not given to the rheumatic patients until the third to the fifth week after the onset of the streptococcic infections; hence the conditions were different from those in the aforementioned investigations.[35] The observations would have been better controlled if a rheumatic group who were not receiving salicylates had been utilized, but for obvious reasons this was impractical.

Such suggestions, as the figures herein presented may indicate, could only be brought out from an analysis of groups and not from the findings in any one person, especially when the manifestations of rheumatic fever may appear any time within a period of one to eight weeks after the precursory streptococcic infection. Possibly with the development of further knowledge of streptococcic antigens and of new technics, these suggestions will lead to further exploration.

SUMMARY AND CONCLUSIONS

1. One hundred and sixty-nine acute hemolytic streptococcic infections of the upper respiratory tract in 153 rheumatic and nonrheumatic patients were studied.

A. Group A streptococci caused 163 of these infections, group C caused 6 and group G caused 1. No cross infections occurred, and in no case did the same serologic type of group A streptococcus cause two separate infections in the same patient.

B. One hundred and two of the infections were uncomplicated, 29 were followed by purulent complications due to the same strain causing the original infection and 38 were followed by rheumatic fever. Four of these 38 were also followed with purulent complications.

C. Rheumatic recurrences developed seventeen times (31.4 per cent) as a result of 54 streptococcic infections in 39 previously rheumatic subjects. On the other hand, primary attacks of rheumatic fever resulted from 21 (18.3 per cent) of the 115 streptococcic infections suffered by 114 previously nonrheumatic subjects.

D. Rheumatic manifestations followed only those infections of the upper respiratory tract which were due to group A hemolytic streptococci; among these no special serologic type of streptococcus was found associated either with rheumatic fever or with purulent complications of the original infections. None of the 7 infections due to streptococci of groups C and G led to

rheumatic sequelae, although 5 of these occurred in patients who had previously had rheumatic fever.

2. Determinations of antistreptolysin O and antifibrinolysin were done weekly on all patients. Seventy-one of these patients (with 83 infections) were tested for other immune responses, i.e., type-specific bacteriostatic antibodies and precipitins against type-specific M, group-specific C and streptococcic nucleoprotein. The serums of these 71 patients were also tested for C-reactive protein, and serums from 9 patients with rheumatic fever were examined for "phase" precipitins.

A. Significant rises of antistreptolysin O occurred in 77 per cent, of antifibrinolysin in 73 per cent, of bacteriostatic antibodies in 76 per cent, of anti-M precipitins in 64 per cent and of C-reactive protein in 71 per cent of the 83 infections. Similar results were obtained for the antistreptolysin O and antifibrinolysin titers of patients in the complete series of 153. No definite phase precipitins could be demonstrated in the 9 patients examined. Three different streptococcic antibodies were demonstrable in the serum of 50 per cent of the patients in the series of 83 infections, and one or more of the several antibodies investigated were found during convalescence in every patient.

B. The patients with rheumatic fever as a group exhibited rises in antistreptolysin O, antifibrinolysin and type-specific antibodies more frequently than did the group of patients who had purulent complications or who made uneventful recoveries. Patients with purulent complications, however, exhibited the greatest increases in antistreptolysin O titer.

C. A slight delay in the beginning of the rise of type-specific bacteriostatic antibodies and anti-M precipitins, as well as a similar delay until the maximal level of antistreptolysin O was reached, was observed in the group of patients with rheumatic fever as compared with the nonrheumatic groups.

3. From this study it is evident that at present no single pattern of antibody response can be used to diagnose the existence of rheumatic fever in any single person.

1. (*a*) McEwen, C.; Bunim, J. J., and Alexander, R. C.: Bacteriologic and Immunologic Studies in Arthritis: II. Results of Various Immunologic Tests in Different Forms of Arthritis, J. Lab. & Clin. Med. **21:** 465, 1936. (*b*) Spink, W. W., and Keefer, C. S.: Studies of Hemolytic Streptococcal Infections: II. The Serological Reactions of the Blood During Erysipelas, J. Clin. Investigation **15:** 21, 1936. (*c*) Mote, J. R., and Jones, T. D.: Studies of Hemolytic Streptococcal Antibodies in Control Groups, Rheumatic Fever, and Rheumatoid Arthritis: II. The Frequency of Antistreptolysin "O," Antifibrinolysin, and Precipitating-Antibody Responses in Scarlet Fever, Hemolytic Streptococcal Infections, and Rheumatic Fever, J. Immunol. **41:** 61, 1941; III. The Magnitude of Anti-Streptolysin "O," Antifibrinolysin, and Precipitating-Antibody Responses; the Persistence of the Antibodies and Variations in Antistreptolysin

"O" Curves in Scarlet Fever. Hemolytic Streptococcal Infections and Rheumatic Fever, ibid. **41:** 87, 1941.

2. Todd, E. W.: Antihaemolysin Titres in Haemolytic Streptococcal Infections and Their Significance in Rheumatic Fever, Brit. J. Exper. Path. **13:** 248, 1932.

3. Tillett, W. S.; Edwards, L. B., and Garner, R. L.: Fibrinolytic Activity of Hemolytic Streptococci: The Developmnet of Resistance to Fibrinolysis Following Acute Hemolytic Streptococcus Infections, J. Clin. Investigation **13:** 47, 1934.

4. Hadfield, G.; Magee, V., and Perry, C. B.: The Lysis of Fibrin by Streptococci: Its Application to the Problems of Rheumatic Infection in Children, Lancet **1:** 834, 1934.

5. Todd, E. W.; Coburn, A. F., and Hill, A. B.: Antistreptolysin S Titres in Rheumatic Fever, Lancet **2:** 1213, 1939.

6. (*a*) Swift, H. F., and Hodge, B. E.: Type Specific Anti-M Precipitins in Rheumatic and Non-Rheumatic Patients with Hemolytic Streptococcal Infections, Proc. Soc. Exper. Biol. & Med. **34:** 849, 1936. (*b*) Coburn, A. F.: Observations on the Mechanism of Rheumatic Fever, Lancet **2:** 1025, 1936.

7 (*a*) Todd, E. W.: The Influence of Sera Obtained from Cases of Streptococcal Septicaemia on the Virulence of the Homologous Cocci, Brit. J. Exper. Path. **8:** 361, 1927. (*b*) Hare, R.: Alterations in the Bactericidal Power of the Blood Which Occur During Haemolytic Streptococcal Infections in the Puerperium, J. Path. & Bact. **41:** 61, 1935. (*c*) Kuttner, A. G., and Lenert, T. F.: The Occurrence of Bacteriostatic Properties in the Blood of Patients After Recovery from Streptococcal Pharyngitis, J. Clin. Investigation **23:** 151, 1944. (*d*) Rothbard, S.: Bacteriostatic Effect of Human Sera on Group A Streptococci: I. Type-Specific Antibodies in Sera of Patients Convalescent from Group A Streptococcal Pharyngitis, J. Exper. Med. **82:** 93, 1945. Spink and Keefer.[1b]

8. Walker, D. W.: Application of the Technique of Slide Agglutination of Hemolytic Streptococci to Human Sera, Proc. Soc. Exper. Biol. & Med. **48:** 338, 1941. Rantz, L. A.; Kirby, W. M. M., and Jacobs, A. H.: Group A Hemolytic Streptococcus Antibodies: Griffith Type Agglutinin and Antistreptolysin Titers in Normal Men and in Acute Infections, J. Clin. Investigation **22:** 411, 1943.

9. Diefendorf, H. W.: A Method for Detecting in Human Serum Protective Bodies Against Hemolytic Streptococci, Proc. Soc. Exper. Biol. & Med. **48:** 56, 1941.

10. Kuttner, A. G., and Krumwiede, E.: Observations on the Effect of Streptococcal Upper Respiratory Infections on Rheumatic Children: A Three Year Study, J. Clin. Investigation **20:** 273, 1941.

11. Lancefield, R. C.: The Antigenic Complex of Streptococcus Haemolyticus: I. Demonstration of a Type-Specific Substance in Extracts of Streptococcus Haemolyticus, J. Exper. Med. **47:** 91, 1928. Swift, H. F.; Wilson, A. T., and Lancefield, R. C.: Typing Group A Hemolytic Streptococci by M Precipitin Reactions in Capillary Pipettes, J. Exper. Med. **78:** 127, 1943.

12. Todd, E. W.: Antigenic Streptococcal Hemolysin, J. Exper. Med. **55:** 267, 1932.

13. Hodge, B. E., and Swift, H. F.: Varying Hemolytic and Constant Combining Capacity of Streptolysins: Influence on Testing for Antistreptolysins, J. Exper. Med. **58:** 277, 1933.

14. Boisvert, P. J.: The Streptococcal Antifibrinolysin Test in Clinical Use, J. Clin. Investigation **19:** 65, 1940.

15. Fuller, A. T.: The Formamide Method for Extraction of Polysaccharides from Haemolytic Streptococci, Brit. J. Exper. Path. **19:** 130, 1938.

16. Lancefield, R. C.: The Immunological Relationships of Streptococcus Viridans and Certain of Its Chemical Fractions: I. Serological Reactions Obtained with Antibacterial Sera, J. Exper. Med. **42:** 377, 1925.

17. Tillett, W. S., and Francis, T., Jr.: Serological Reactions in Pneumonia with a Non-Protein Somatic Fraction of Pneumococcus, J. Exper. Med. **52:** 561, 1930.

18. With pneumococcus C polysaccharide extract 0.15 cc. of antigen was mixed with an equal volume of undiluted test serum.

19. For the phase precipitin test, 0.1 cc. of antibody serum (phase III serum in acute stage of rheumatic fever) was layered over 0.1 cc. of antigen serum (phase II, prerheumatic or poststreptococcic stage).

20. Coburn, A. F., and Pauli, R. H.: A Precipitinogen in the Serum Prior to the Onset of Acute Rheumatism, J. Exper. Med. **69:** 143, 1939.

21. The blood was obtained from children with noninfectious orthopedic disturbances in the New York Orthopedic Hospital, the Hospital for Special Surgery and the New York Hospital through the assistance of Dr. Philip D. Wilson, Dr. Alan De Forest Smith and Dr. S. Z. Levine.

22. Watson, R. F.; Rothbard, S., and Swift, H. F.: The Relationship of Postcarlatinal Arthritis and Carditis to Rheumatic Fever, J. A. M. A. **128:** 1145 (Aug. 18) 1945.

23. Watson, R. F.; Schwentker, F. F.; Fetherston, J. E., and Rothbard, S.: Sulfadiazine Prophylaxis in an Epidemic of Scarlet Fever, J. A. M. A. **122:** 730 (July 10) 1943.

24. Since these tests were made, Kaplan has recently devised a quantitative serologic method for the estimation of serum antifibrinolysin which possesses a number of advantages over the plasma test (Kaplan, M. H.: J. Clin. Investigation **25:** 347, 1946).

25. Perlman, E.; Bullowa, J. G. M., and Goodkind, R.: An Immunological and Electrophoretic Comparison of the Antibody to C Polysaccharide and the C Reactive Protein of Acute Phase Serum, J. Exper. Med. **77:** 97, 1943.

26. Ash, R.: Non-Specific Precipitins for Pneumococcic Fraction C in Acute Infections, J. Infect. Dis. **53:** 89, 1933. Abernethy, T. J., and Avery, O. T.: The Occurrence During Acute Infections of a Protein Not Normally Present in the Blood: I. Distribution of the Reactive Protein in Patients' Sera and the Effect of Calcium on the Flocculation Reaction with C Polysaccharide of the Pneumococcus, J. Exper. Med. **73:** 173, 1941. Tillett and Francis.[17]

27. These percentages would probably have been increased if antiserum prepared in rabbits to C-reactive protein from human sources (Macleod, C. M., and Avery, O. T.: J. Exper. Med. **73:** 191, 1941) had been used instead of the "C" polysaccharide.

28. Escherich, T., and Schick, B., Scharlach, Vienna, A. Hölder, 1912.

29. Schlesinger, B.: The Relationship of Throat Infection to Acute Rheumatism in Childhood, Arch. Dis. Childhood **5:** 411, 1930.

30. Coburn, A. F., and Pauli, R. H.: Studies on the Relationship of Streptococcus Hemolyticus to the Rheumatic Process: III. Observations on the Immunological

Responses of Rheumatic Subjects to Hemolytic Streptococcus, J. Exper. Med. **56:** 651, 1932.

31. Wedum, A. G., and Wedum, B. G.: Serum Precipitation Reaction in Rheumatic Fever and in Other Conditions, Proc. Soc. Exper. Biol. & Med. **61:** 432, 1946.

32. Reyersbach, G.; Lenert, T. F., and Kuttner, A. G.: An Epidemic of Influenza B Occurring in a Group of Rheumatic Children Concurrent with an Outbreak of Streptococcal Pharyngitis: Clinical and Epidemiological Observations, J. Clin. Investigation **20:** 289, 1941. Green, C. A.: Epidemiology of Haemolytic Streptoccal Infections in Relation to Acute Rheumatism: III. Comparative Incidence of Various Infections and Acute Rheumatism in Certain Training Centers, J. Hyg. **42:** 380, 1942. Rantz, L. A.; Boisvert, P J., and Spink, W. W.: Etiology and Pathogenesis of Rheumatic Fever, Arch. Int. Med. **76:** 131 (Sept.) 1945.

33. Bailey, J. H.: The Types of Hemolytic Streptococci Found in Scarlet Fever Patients and in Throats of Grammar-School Children, Am. J. Hyg. **29:** 107, 1939. Colebrook, L.; Elliott, S. D.; Maxted, W. R.; Morley, C. W., and Mortell, M.: Infection by Non-Hemolytic Group A Streptococci, Lancet **2:** 30, 1942. Lancefield, R. C., and Stewart, W. A.: Studies on the Antigenic Composition of Group A Hemolytic Streptococci: II. The Occurrence of Strains in a Given Type Containing M but No T Antigen, J. Exper. Med. **79:** 79, 1944. Herbert, D., and Todd, E. W.: The Oxygen-Stable Haemolysin of Group A Haemolytic Streptococci (Streptolysin S), Brit. J. Exper. Path. **25:** 242, 1944. Studies on Streptococcal Fibrinolysis. V. The in Vitro Production of Fibrinolysin by Various Groups and Types of Beta Hemolytic Streptococci; Relationship to Antifibrinolysin Production, Commission on Acute Respiratory Diseases, J. Exper. Med. **85:** 441, 1947.

34. Thomas, R. A.: Precipitation and Agglutination Tests with the Hemolytic Streptococcus: Titration of "M" and "T" Anti-bodies in Human Sera, Science **100:** 552, 1944.

35. Swift, H. F.: The Action of Sodium Salicylate upon the Formation of Immune Bodies, J. Exper. Med. **36:** 735, 1922. Derick, C. L.; Hitchcock, C. H., and Swift, H. F.: The Effect of Anti-Rheumatic Drugs on the Arthritis and Immune Body Production in Serum Disease, J. Clin. Investigation **5:** 427, 1928. Homburger, F.: Sodium Salicylate Inhibiting Anti-Rh Immunization in Animals, Proc. Soc. Exper. Biol. & Med. **61:** 101, 1946.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, June 1, 1949,
Vol. 89, No. 6, pp. 687–698]

INDUCTION OF CARDIAC LESIONS, CLOSELY RESEMBLING THOSE OF RHEUMATIC FEVER, IN RABBITS FOLLOWING REPEATED SKIN INFECTIONS WITH GROUP A STREPTOCOCCI

BY GEORGE E. MURPHY, M.D.,* AND HOMER F. SWIFT, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research*)

PLATES 37 TO 42

(Received for publication, March 10, 1949)

Elucidation of the rôle of group A streptococci in the pathogenesis of rheumatic fever might be furthered if a host alteration closely simulating this disease could be induced in laboratory animals infected with these microorganisms; but to date efforts in this direction have failed. This failure possibly stems from one or more factors: (1) lower animals may be incapable of developing the disease; (2) the streptococci employed may have been unable to induce the characteristic host alterations; (3) the experimental conditions may have been unsuitable.

Because a spontaneous disease closely resembling rheumatic fever has not been found in lower animals, its experimental induction might be impossible. The streptococci usually pathogenic for animals belong to serological groups other than A, whereas group A streptococci are chiefly pathogenic for man, and in so far as is known this group comprises the only streptococci that induce the respiratory infections preceding rheumatic fever; the host-parasite relationships among lower animals and streptococci may not be reflected in a rheumatic fever-like state. In experimental streptococcal infections, single strains have usually been employed; but valid data indicate that successive group A streptococcal infections in one person are probably caused by different serological types (1, 2). Rheumatic fever, moreover, occurs among patients in an age period and under conditions which make it probable that they had experienced one or more previous streptococcal infections.

In investigating possible relationships between rheumatic fever and various states of altered reactivity induced experimentally in animals infected with streptococci, workers in this laboratory observed the following phenomena: (*a*) focal infections of rabbits with viridans, group A or C streptococci resulted in the development of clear cut cutaneous and general hyperreactivity to the homologous infecting strain (3, 4), which was markedly enhanced by frequently repeated minute intracutaneous inoculations (5); (*b*) intravenous injections of living viridans streptococci (6), or of heat-killed vaccines of group A or C streptococci (5) induced in rabbits a state of diminished cutaneous reactivity

* This work was done during the tenure of a Life Insurance Medical Research Fellowship.

to inoculation with homologous strains; (*c*) these immunized animals simultaneously showed cutaneous hyperreactivity to strains belonging to heterologous groups (4, 7); (*d*) some rabbits immunized intravenously or subcutaneously with heat-killed vaccines of one type of group A streptococci developed decreased skin reactivity to intracutaneous inocula of the same type, but simultaneously showed greater than normal cutaneous reactivity to minute intracutaneous inocula with heterologous types, and the same often held true when the preliminary immunization was induced by repeated skin infections with a strain of a very rabbit-virulent group A streptococcus (8), and the state of cutaneous hyperreactivity was brought out far more clearly with high dilutions of the challenging inocula than with low dilutions; (*e*) rabbits resting 2 or more months from immunization with viridans, group A or C streptococci became cutaneously and systemically hyperreactive to the homologous strain of streptococci previously injected (7). The following information was also available: Rheumatic fever patients develop type-specific antibodies to the group A streptococcus inducing the nasopharyngeal infection preceding a rheumatic fever attack (1, 2); rheumatic fever patients, as a rule, were found to be hyperreactive to viridans and group A streptococcal nucleoproteins (9) and to group A streptococcal vaccines (10). It was later assumed that this hyperreactive state was induced by recurring focal (nasopharyngeal) infections with a succession of different serological types of group A streptococci.

These observations suggested that a rheumatic fever–like state might be induced in animals by successive focal group A streptococcal infections, each caused by a serological type heterologous to those previously employed. This communication reports the first testing of this hypothesis.

Methods

Because it was desirable to test a relatively large group of animals, and as considerable information was available concerning the reactivity of rabbits to streptococcal infections, this species was chosen. New Zealand Reds and a cross-breed designated hare brown, all bred in the Rockefeller Institute, were usually employed; occasionally chinchilla and other varieties were tested. All rabbits were fed approximately 560 gm. of Rockland rabbit ration pellets within each week. Animals with skins which mostly remained bare for considerable periods after close clipping were preferred; and such clipped sites were used primarily; but after repeated inoculations it sometimes became difficult to find very suitable skin, and coarsely hairy areas had to be inoculated.

The group A streptococci employed all exhibited matt or mucoid colony forms in 18 to 24 hours growth on moist rabbit blood agar; and were shown to produce large amounts of type-specific M protein in Todd-Hewitt broth made with neopeptone. Mostly they had only moderate virulence for rabbits, but even in this respect there was considerable variation, both among the various types employed and in different subcultures of single strains. Efforts to increase virulence by rabbit passage have been only moderately successful.

Sixteen to 20 hour Todd-Hewitt neopeptone broth cultures were serially diluted in tenfold steps with broth, and the inocula in 0.1 cc. volume, containing between 10^{-8} and 10^{-2} cc. of the original culture, were injected into closely clipped skin of right and left gluteal, lumbar, thoracic, or shoulder areas.. Ten times more cocci were injected on the right side than on the

left. In the original groups of animals, the 2nd to 4th successive focal cutaneous infections were set up in the same well healed, but scarred, gluteal sites. Subsequently, because of re-

TABLE I

Rabbit 70-58—New Zealand ♀

Infections					Course of infections				
Date	Strepto-coccus type	Inocula	Skin site	Skin reaction	Date	Weight	ESR	ASO units	Remarks
1946					*1946*	*kg.*	*mm./hr.*		
7/17	1	10^{-8}	Gl	N	7/17	3.0			
8/28	11	10^{-5}	Gl		8/19	3.1			
10/ 8	17	10^{-6}	Gl		10/30		1		
11/12	13	10^{-4}	Gl	Initially	11/12	3.8		<25	
11/15	13	10^{-4}	Gl	>N; <N	11/16		1		
11/19	13	10^{-3}	Gl	in few	11/25		1	25	
11/25	13	10^{-3}	Gl	days	11/28		1	25	
1947					*1947*				
1/13	3	10^{-4}, 10^{-6}	Gl;	Th—>N	1/10	3.7			
					1/18		1	25	
					1/20	3.8			
4/18	19	10^{-4}, 10^{-5}	Gl;	Sh—>N	4/16	4.0			
					4/25		1	50	
5/28	17	10^{-5}, 10^{-6}	Gl;	Lu—<N	5/27			25	
					6/ 7		1	25	
9/16	1	10^{-4}, 10^{-5}	Gl;	Th—≫N	9/19				
					9/22				Anorexia
					9/25		130	400	"
					9/26		47	>1000	"
					9/27		76		" ‡
					9/29				Died§

N, average cutaneous response of normal control rabbits to intracutaneous inoculation with streptococci.

Gl indicates gluteal; Lu, lumbar; Th, thoracic; Sh, shoulder.

* Erythema over right knee.

‡ Negative blood culture.

§ Autopsy blood culture negative.

ESR, erythrocyte sedimentation rate (Westergren).

ASO, antistreptolysin O titer.

E.S.R. of normal rabbits, 1 to 2 mm. per 1 hour and 2 to 4 mm. per 2 hours.

sults recorded below, each reinfection, usually with a type of streptococcus not previously injected, included 4 areas: right and left scarred gluteal skin sites and a right and left skin site least likely to have been locally inflamed in previous infections.

All skin lesions were measured daily until recession was demonstrated. The condition of the rabbits was observed; and their weights were recorded at suitable intervals. Blood was obtained from the ear veins for serum which was refrigerated and later tested for antistreptolysin O and antistreptokinase content, and for precipitin reactions with extracts of homologous and heterologous types of group A streptococci. These data furnished a rough index of the serological responses to the infections. During the earlier experiments erythrocyte sedimentation rate determinations (Westergren method) were made once or twice during the fortnight following the inoculations; but later both erythrocyte sedimentation rate determinations and leucocyte counts were made twice or thrice weekly until they were approximately normal or until the animal died or was sacrificed

TABLE II

Rabbit 71-77—Hare Brown ♂

Infections					Course of infections					
Date	Streptococcus type	Inocula	Skin site	Skin reaction	Date	Weight	ESR	WBC 1000	ASO units	Remarks
1947					*1947*	*kg.*	*mm./hr.*			
5/ 8	19	10^{-4}, 10^{-6}	Gl;	Sh—>2 other norms	5/8	2.7				
6/20	1	10^{-4}, 10^{-6}	Gl;	Lu—≪N	6/20				<25	
9/16	1	10^{-4}, 10^{-6}	Gl;	Th—≪N						
11/ 6	London	10^{-3}, 10^{-4}	Gl;	Th—=N	11/ 4				<25	
					11/19		3		75	
1948					*1948*					
					1/16		1	7.7	25	
2/ 6	3	10^{-3}, 10^{-4}	Gl;	Th—<N	2/ 6	3.4				
					2/14	2.8	152	10.8	600	Anorexia, marked, cardiac irregularity· died*

* Negative blood culture ante- and postmortem.

Where indicated, blood cultures were made from living rabbits with blood obtained from ear veins and placed both in Todd-Hewitt neopeptone blood broth and on rabbit blood agar plates. Blood obtained postmortem from the inferior vena cava of all dead rabbits was similarly cultured; and streptococci recovered were identified serologically.

During the first 4 to 5 months' experimentation it was found that successive monthly to bimonthly inoculations with streptococci of different serological types into the same gluteal skin sites usually induced progressively smaller local lesions than those which followed similar inoculation in comparable skin of normal controls; but in their previously uninfected (*e.g.*

thoracic) skin the same sized inoculum almost invariably induced greater local inflammation than in their multiply infected gluteal skin or in the thoracic skin of normal controls (Table I;

TABLE III

Rabbit 71-80—Hare Brown ♀

Infections					Course of infections						
Date	Streptococcus type	Inocula	Skin site	Skin reaction	Date	Weight	ESR 1 hr.	ESR 2 hr.	WBC 1000	ASO units	Remarks
1947					*1947*	*kg.*	*mm.*				
5/28	6	10^{-3}, 10^{-4}	Gl;	Lu—>2 other norms	5/28	3.4					
					6/ 7	2.7				700	
6/20	1	10^{-4}, 10^{-5}	Gl;	Th—≪N	6/20	2.8				<25	
9/16	1	10^{-4}, 10^{-5}	Gl;	Th—<N							
					9/30		1			100	
11/ 6	London	10^{-3}, 10^{-4}	Gl;	Sh—=N	11/ 3					100	
					11/14	3.3					
					11/18	3.2	1			75	
1948					*1948*						
					1/19	3.4	1		16.2	25	
2/ 6	3	10^{-3}, 10^{-4}	Gl;	Th—<N							
					2/16	3.4	4		19.3	50	
					2/19		2		14.2	75	
					2/25		3		13.5	25	
					5/26	3.5					
9/21	9	10^{-3}, 10^{-4}	Gl;	Th—=N	9/21	3.5	1		18.0		
9/22	9	10^{-2}, 10^{-3}	Lu		9/22				22.5		
					9/23		5	11		<25	
					9/24	3.1	7	16	18.9	<25	
					9/26	2.9					
					9/27	2.7	29	56	30.1	50	
					9/28	2.7	33	54	21.8	50	Anorexia
					9/29		49	80		75	Diarrhea
					10/ 1	2.3	45	88	19.5	200	
					10/ 2	2.4				100	Sacrificed§

* Guards hind legs.

‡ Negative blood culture.

§ Autopsy blood culture negative.

January, 1947). It was, therefore, obvious that new areas were requisite to obtain a rough approximation of an animal's cutaneous reactivity to successive inoculations. Furthermore, because of this finding that succeeding streptococcal skin inflammation was likely to be more

intense in previously uninflamed skin than in scarred sites, it seemed possible that streptococci might survive longer and effect more sustained local infection in fresh skin than in scarred areas; and that from the larger inflammatory zones more toxic material might be elaborated and absorbed than from the small lesions.

Variations in the inoculation procedures are illustrated in Tables I, II, and III. Occasionally, as in November, 1946 (Table I), or in September, 1948 (Table III), repeated intracutaneous inoculations with the same type of streptococcus were given within a few days in attempts to enhance the infectious stimulus of lowly virulent strains. In other instances, as with type 1 (Tables I, II, and III), the same type was reinjected after a relatively long interval; but generally each successive inoculation in a given animal was with a type heterologous to those previously used to infect a given animal.

Autopsies were performed as soon after death as possible; those on sacrificed rabbits were carried out immediately after exitus, usually effected with intravenous sodium nembutal. Tissues were fixed in Zenker-acetic acid, and sections cut from paraffin blocks were stained with hematoxylin and eosin, Giemsa, Weigert-hematoxylin and eosin, Masson's trichrome, Mallory's aniline blue, and where indicated with Gram-Weigert and malachite green-acridine red (11), a technique applicable to Zenker-fixed tissues, whereas the Unna-Pappenheim methyl green-pyronine technique requires alcohol fixation.

RESULTS

After sustaining 2 to 10 infections with streptococci of different serological types within 3 to 20 months, some rabbits sickened and showed various combinations of the following signs and symptoms: elevated erythrocyte sedimentation rates for 1 to 2 weeks; leucocytosis; anorexia; weight loss; postexertional dyspnea; occasional transient pulmonary rales; tachycardia; and in a few instances, definitely irregular cardiac rhythm. Many of these rabbits recovered; a portion were sacrificed within 10 to 14 days following their last infection while exhibiting definite symptoms, leucocytosis, and elevated erythrocyte sedimentation rates higher than were occurring in normal controls; in several rabbits, however, a severe illness developed following the last streptococcal infection and terminated fatally, whereas some of the normal controls survived the same streptococcal infection. A few in the fatal group died within 2 to 5 days following the last infection (even though normal controls in some instances survived) and in all except one of these rapidly fatal cases, streptococcal bacteremia was established at autopsy. In about half of a group of rabbits dying spontaneously between 6 and 14 days after the last infection, streptococcal bacteremia was demonstrated at autopsy; in the other rabbits of this group, however, streptococci could not be cultured from the blood either before death or at autopsy.

In the hearts of the successively infected rabbits which had sickened and succumbed, and of those sacrificed while sick, there have been found on microscopic examination focal alterations in the connective tissue framework in blood vessel adventitia, valves, mural endocardium, epicardium, and in the myocardial interstitium. Many collagen fibers in these sites are swollen; some are intensely eosinophilic, others stain poorly; some swollen collagen fibers stain entirely, whereas others stain in patchy fashion like fibrin with both Masson's trichrome and Mallory's connective tissue techniques. Arranged about and interspersed

in fields of swollen "fibrinoid" collagen are nodular collections of large, irregularly shaped cells, often with abundant, finely granular basophilic cytoplasm which takes a smudgy red color with the malachite green–acridine red stain. Often these cells have very indistinct outlines; some have long streamer-like cytoplasmic processes which gradually fade into the contiguous areas. The vesicular nuclei, single or multiple, are variously shaped, and have sharply defined membranes. Clumping of chromatin often leaves the rest of the nucleus clear. Some nuclei are pyknotic. Cells with multiple, centrally placed nuclei, 2 to 10 in number, occur in greatest profusion in the mitral and aortic sulci and rings and in the endocardium (Fig. 3). The lesions also contain many cells of the Anitschkow myocyte type, and occasionally small round cells and polymorphonuclear leucocytes, both pseudo- and true eosinophiles. The sites of predilection for the occurrence of these nodular granulomata in most hearts are endocardial, subendocardial, and blood vessel adventitia and paraadventitia. These adventitial lesions are by no means limited to the roots of the valves, but at times are conspicuously present throughout the hearts, particularly in the left ventricle and interventricular septum.

In some hearts the granulomata occur in the loose myocardial interstitium unassociated with arteries or veins but, in most instances, with capillaries. In agreement with Gross (12) the latter are designated "myocardial granulomata" to distinguish them from granulomata associated with other cardiac structures. Interstitial valvulitis, most marked in the middle of the cusps, has occurred commonly in the mitral and aortic valves and also in the right auriculoventricular valve; these areas beneath the line of closure show edema of varying intensity and cellular components like those in the submiliary nodules. Marked proliferation of mitral and aortic valvular endocardial and subendocardial cells occurs in several hearts to create many layered palisades containing numerous multinucleated giant cells dispersed in swollen or "fibrinoid" collagen (Figs. 1 and 2). These lesions are occasionally limited to the sulci, but are also found often on both surfaces of the valve and of the chordae tendineae (Fig. 10). At times the most superficially palisaded cells have apparently undergone necrosis and conversion into acellular material that stains like fibrin. The latter phenomenon was most marked in rabbits dying spontaneously within 2 weeks after the final infection. On no valves were there seen, macroscopically, rows of fine verrucae along the lines of closure. In the gross, however, the mitral valve of several rabbits showed along the line of closure a row of fine discrete opalescent elevations usually more marked on the aortic leaflet. Microscopically these elevations consist of interstitial edema and valvulitis which in some instances are more intense than in the neighboring tissues. Occasionally larger fine, firm white nodules projecting from the surface of the valve were visible. Foci of frankly "fibrinoid" collagen[1] are seen in auricular (Fig. 12) and ventricu-

[1] The expression "frankly fibrinoid collagen" indicates that the altered collagen stains unequivocally like fibrin with Mallory's connective tissue and Masson's trichrome techniques.

lar epicardium in association with proliferated epicardial and subepicardial elements. These patches of epicarditis are microscopic in size; and no extensive plastic pericardial exudate has been detected in the gross.

Granulomata in the compact paravascular connective tissue differ in architectural configuration from the "myocardial granulomata" in the looser tissue between muscle bundles. There are submiliary granulomata closely resembling the coronal (Fig. 6), reticular (Figs. 4 and 7), and mosaic (Figs. 8, 9, and 11) types of Aschoff bodies described by Gross in human rheumatic hearts (12); and in the left ventricle and interventricular septum of a few rabbit hearts several myocardial granulomata are often seen in a low power field; but in no rabbit dying spontaneously or sacrificed within 2 weeks after final infection, have there been found well developed polarized or fibrillar types of granulomata. Gross considered the peculiarly shaped and arranged cells in such Aschoff bodies to represent terminal metamorphosis of the rheumatic granuloma cells into fibroblasts. Damage to myocardium adjacent to granulomata has been prominent, and has ranged from swelling and vacuolation of the myofibers and vesiculation of their nuclei to complete dissolution and replacement by scar. Occasionally apparent fusion of neighboring granulomata combined with extensive adjacent myocardial destruction and connective tissue replacement has resulted in macroscopically visible lesions in stained sections of left ventricle and papillary muscles.[2]

The coronary arterial system is variously altered. Fairly commonly there is marked intimal hyperplasia and elastification involving chiefly small arteries and arterioles. A well developed intimal musculoelastic hyperplastic lesion occurs in several rabbits. In the hearts of two rabbits there is found marked ramification of fibrinoid material throughout or surrounding the wall of a small artery or capillary (Fig. 5). Interspersed in and arranged about the extension of this intensely eosinophlic material into the tissue adjacent to the vessel are granuloma cells of the type found in the previously described rabbit granulomata. This vascular lesion closely resembles that described in rheumatic human hearts by Pappenheimer and Von Glahn (13). Panarteritis of the so called "allergic" or periarteritis nodosa type is conspicuously absent in the hearts of all intracutaneously infected rabbits. Verrucous and polypoid endarteritis are occasionally present. In the intima and immediately subadjacent media of the aorta near its root there occasionally is seen a lesion comparable with that in the valve sulci and closely resembling that described by Pappen-

[2] A striking increase in size of the adrenal glands occurred in rabbits dying, or sacrificed while sick, following the last of several intracutaneous streptococcal infections. Microscopically hyperplasia, hypertrophy, and necrosis of fascicular zone cells are seen. There is striking correlation between the degree of macroscopic enlargement of the fascicular zone of the adrenal cortex and the occurrence of myocardial granulomata. Detailed data concerning these observations will be presented shortly.

heimer and Von Glahn (13) in human rheumatic aortitis. Occasionally there are foci of clearly defined fibrinoid collagen in the adventitia of the root and first portion of the aorta. These lesions will be illustrated later.

Neither bacteria nor any structures resembling inclusion bodies have been seen in the above described lesions stained according to Gram-Weigert or Giemsa techniques. There has, moreover, been no calcification of the myocar-

TABLE IV

Rabbit groups	No.	Bacteremia	Acute rheumatic fever-like cardiac lesions	Myocardial scars or healed arteritis of rheumatic type
1. Normal rabbits	8	—	0	0
2. I.v. vaccine (group A or C streptococci)	8	—	0	0
3. Dying 1–18 days after 1 i.v. infection	20	20	0*	0
4. Sacrificed 1 and 4 mos. after 1 i.v. infection	2	0	0	0
5. Sacrificed within 1 mo. after 1 i.c. infection	4	0	0	0
6. Dying within 2 wks. after 1 i.c. infection	13	12	0	0
7. Dying 3 wks. after 1 i.c. infection	1	1	1‡	0
8. Sacrificed 10 to 21 days after last of several i.c. infections	37	0	7	8
9. Dying 2 to 5 days after 2nd i.c. infection	3	2	3§	0
10. Dying 8 to 14 days after last of 5 to 9 i.c. infections	3	0	3	2
11. Dying 5 to 9 days after last of 2 to 9 i.c. infections	7	7	6	3
12. Dying several weeks after last of several i.c. infections	4	0	0	3
Totals	110	42	20	16

i.v., intravenous. i.c., intracutaneous.

* The hearts of two rabbits dying 12 and 18 days, respectively, after one i.v. inoculation show an acute exudative and necrotizing arteritis.

‡ Interstitial valvulitis marked; vascular adventitial and interstitial foci of lymphocytes and plasma cells and occasional foci of young mesenchymal cells in adventitia without demonstrable alteration of collagen; no necrotizing arteritis.

§ Slight interstitial valvulitis only.

dial lesions, a very conspicuous phenomenon in experimental myocarditis induced with filterable viruses.

Controls.—The tissues of rabbits of the same stock and breeds, both normal and subjected to various experimental procedures have been examined at intervals during the investigative period in order to learn whether comparable lesions were occurring in such control animals, for it should be recalled that a peculiar myocarditis was described by Loewe and his coworkers (14) among stocks of rabbits injected with various materials as well as among uninoculated

controls. These workers ascribed these lesions to a spontaneous epidemic in their stock. As indicated in Table IV, enough controls have been examined to eliminate fairly certainly the possibility that such an epidemic existed among the animals we used. The small focal lesions described by Miller (15) in rabbits' hearts have not been encountered among our present stock of experimental animals or controls; hence it seems probable that the cardiac lesions that developed in our animals bear no relationship to previously described spontaneous rabbit myocarditis.

Inspection of Table IV indicates that the cardiac lesions forming the basis for this report have developed only in rabbits that had undergone multiple, successive cutaneous infections with group A streptococci of different types. In most of the animals showing these lesions, ante- and postmortem blood cultures were negative, and with bacterial stains no bacterial cells could be seen in the lesions; hence it seems improbable that the fresh tissue alterations were due to a direct action of streptococcal cells at the site of injury. In some of the animals, there was evidence of terminal streptococcemia, but even so those animals dying acutely with streptococcemia following their first cutaneous infection, or from intravenous inoculation with streptococci have not developed these submiliary granulomata. Similar negative results were found in rabbits repeatedly immunized intravenously with heat-killed group A or C streptococcal vaccines, as well as in those sacrificed after one intracutaneous inoculation. It seems, therefore, that those finally dying with bacteremia following the last of multiple skin infections developed these cardiac lesions (in which repeated bacterial stains have been negative) because the final insult affected tissues peculiarly conditioned by previous focal infections. It seems quite possible that in this group of rabbits, the bacteremia was, in fact, a terminal event.

DISCUSSION

The cardiac granulomata described, which in many respects bear such a striking histopathological similarity to those of human rheumatic fever, have been encountered only in animals that had undergone multiple, successive cutaneous infections with group A streptococci of several different types. It, therefore, seems probable that the relatively long experimental period and the reconditioning that the animals' tissues underwent as a result of several focal infections with different types of group A streptococci were important factors in the pathogenesis of these lesions. In certain respects this experimental procedure follows the pattern encountered in rheumatic fever patients: they have successive infections with different types of group A streptococci, and these infections are usually focalized in the upper respiratory tract and accessory tissues. Because it was impractical to infect rabbits' throats and sinuses repeatedly, and because successive focal infections appeared hypothetically to be important, the rabbits' skin was selected for the repeated insults.

The carditis developed, moreover, following infections with the same microorganisms that have been repeatedly proven to occur in the infections that precede attacks of rheumatic fever in man. This unique sequential relationship could not be demonstrated until Lancefield's system of classification of streptococci was available (16). In rabbits made hyperreactive to viridans, group A and group C streptococci by repeated focal infections and then shocked with intravenous inoculations of homologous streptococci, cardiac lesions of this type were not encountered (17).

It seems expedient to compare the carditis herein described in rabbits with that in animals of the same species with serum disease or subjected to repeated parenteral injections of foreign protein. This will be the subject of a later communication; but available evidence seems to indicate that the over-all histopathological picture in the rabbits repeatedly infected with streptococci bears closer resemblance to that of human rheumatic carditis than does experimental serum disease carditis. The fatal termination within 6 to 14 days, of an illness developing after the last of several focal infections is a phenomenon which, to our knowledge, has not been recorded in rabbits repeatedly injected and shocked with foreign protein.

Among the random samples of rabbits subjected to the described experimental procedure, only a small portion have developed these cardiac lesions. It seems pertinent to mention that only a small proportion of human beings in this geographical area develop rheumatic heart disease, and today an even smaller proportion develop polyarthritis rheumatica. Among subjects who have recovered from previous attacks of rheumatic fever and in rheumatic families, the incidence is considerably higher. There has been no attempt to select specially susceptible stock among the animals used in these experiments.

On the basis of evidence derived from the experiments here reported and from studies of rhuematic fever in man, it seems justified to assume that similar host-streptococcus relationships may be operative and requisite in the pathogenesis of these cardiac lesions in rabbits and rheumatic carditis in man.

SUMMARY

Cardiac lesions, closely resembling those found in rheumatic fever, have developed in rabbits that sickened following multiple, successive skin infections with several serological types of group A streptococci.

It is a pleasure to acknowledge the valuable technical assistance of Miss Jeanne Epstein and Mr. Andrew Littell.

BIBLIOGRAPHY

1. Kuttner, A. G., and Lenert, T. F., *J: Clin. Inv.*, 1944, **23**, 151.
2. Rothbard, S., Watson, R. F., Swift, H. F., and Wilson, A. T., *Arch. Int. Med.*, 1948, **82**, 229.

3. Andrewes, C. H., Derick, C. L., and Swift, H. F., *J. Exp. Med.*, 1926, **44,** 35.
4. Böhmig, R., and Swift, H. F., *Arch. Path.*, 1933, **15,** 611.
5. Derick, C. L., Hitchcock, C. H., and Swift, H. F., *J. Exp. Med.*, 1930, **52,** 1.
6. Swift, H. F., and Derick, C. L., *J. Exp. Med.*, 1929, **49,** 883.
7. Böhmig, R., *Z. Hyg. u. Infektionskrankh.*, 1933, **115,** 406.
8. Hartman, T. L., and Swift, H. F., unpublished observations.
9. Swift, H. F., Hitchcock, C. H., and Derick, C. L., *Proc. Soc. Exp. Biol. and Med.*, 1928, **25,** 312.
10. Swift, H. F., Derick, C. L., and Hitchcock, C. H., *Tr. Assn. Am. Physn.*, 1928, **43,** 192.
11. Hitchcock, C. H., and Ehrich, W., *Arch. Path.*, 1930, **9,** 625.
12. Gross, L., and Ehrlich, J. C., *Am. J. Path.*, 1934, **10,** 467.
13. Pappenheimer, A. M., and Von Glahn, W. C., *Am. J. Path.*, 1927, **3,** 583.
14. Loewe, L., and Lenke, S. E., *J. Exp. Med.*, 1940, **71,** 89.
15. Miller, C. P., Jr., *J. Exp. Med.*, 1924, **40,** 543.
16. Lancefield, R. C., *Harvey Lectures*, 1940–41, **36,** 251.
17. Böhmig, R., *Verhandl. deutsch. path. Ges.*, 1934, **27,** 166.

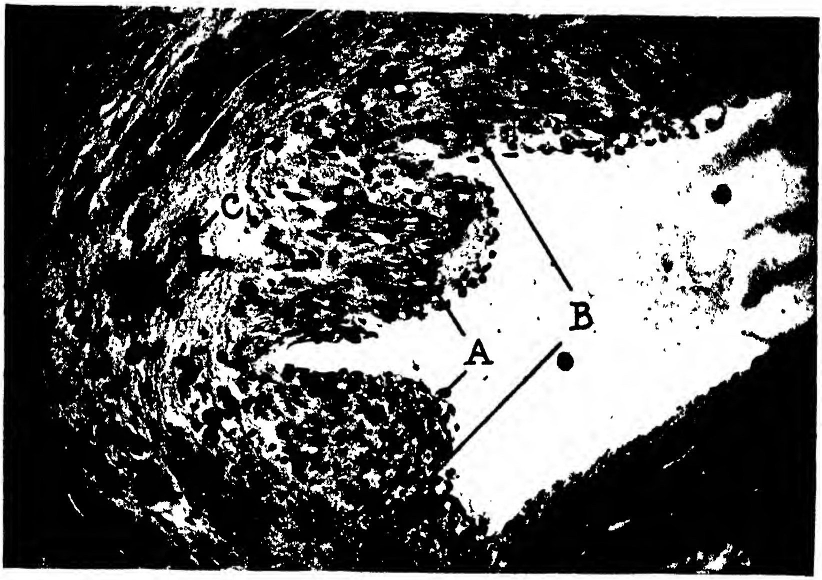
EXPLANATION OF PLATES

The photographs were made by Mr. Julian Carlile and Mr. Richard Carter.

Plate 37

Fig. 1. Rabbit 73-13, sacrificed 15 days after last of 4 infections; no bacteremia at autopsy. *A*, polypoid endo- and subendocardial proliferation (palisade) in mitral sulcus; *B*, external elastic lamella; *C*, focus of frankly fibrinoid collagen. Weigert-hematoxylin and eosin. × 195.

Fig. 2. Rabbit 71-77 (see Table II),—died 8 days after last of 5 infections; no bacteremia ante- or postmortem. *A*, extensive endo- and subendocardial proliferation (palisade) in aortic pocket; *B*, inflammation in annulus; *C*, aortic interstitial valvulitis; *D*, root of aorta. Weigert-hematoxylin and eosin. × 116.

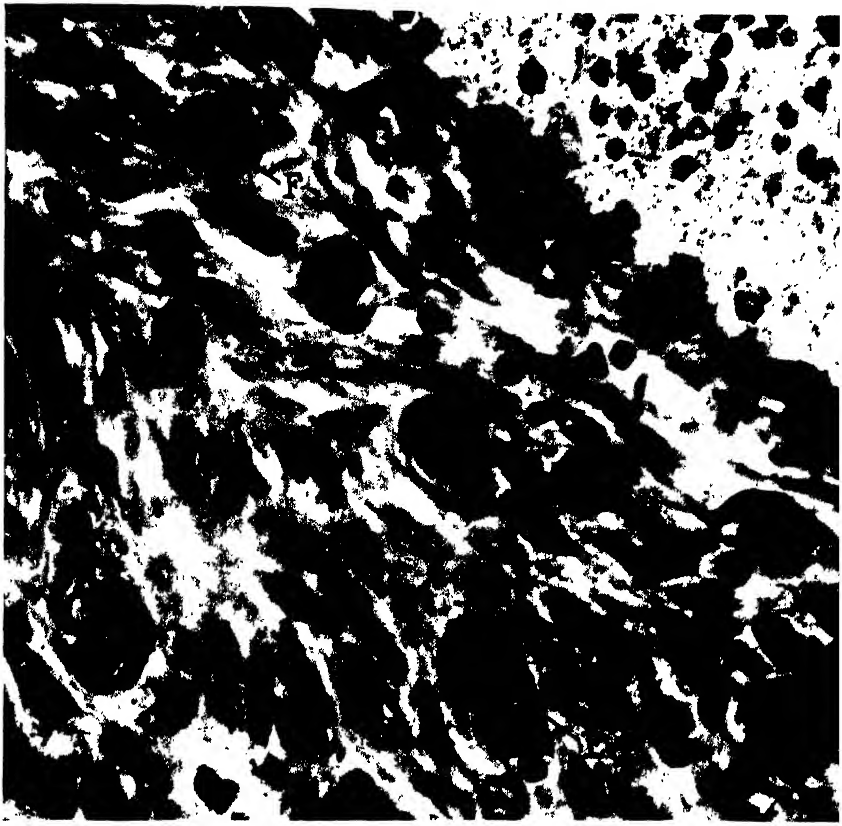

C
A
B


C
2

Plate 38

Fig. 3. Rabbit 71-77, -higher magnification of *A*, Fig. 2; numerous mono- and multinucleated cells, some with bizarre shaped nuclei, basophilic cytoplasm, and indistinct cytoplasmic outline; *E* and *F*, cells with 8 nuclei. Hematoxylin and eosin. × 886.

Fig. 4. Rabbit 71-77, -reticular myocardial granuloma, interventricular septum. *A*, swollen collagen fibers forming interlacing network; collagen framework which assumes a direction roughly parallel with the myocardial bundles; *B*, cell with abundant cytoplasm; necrosis of adjacent myofibers. Weigert-hematoxylin and eosin. × 395.



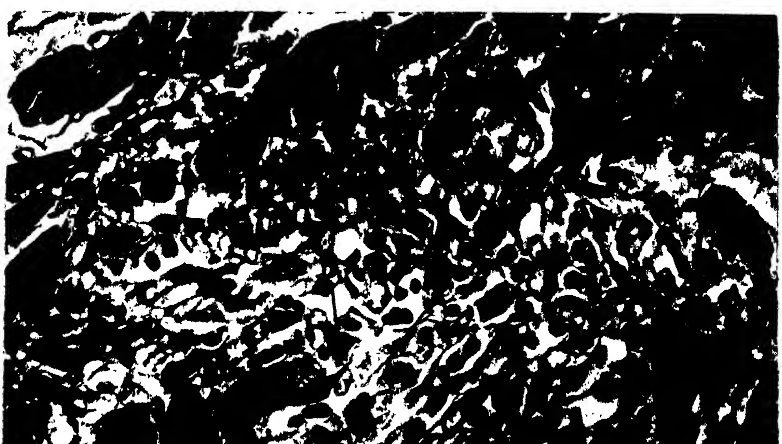


PLATE 39

FIG. 5. Rabbit 71–77, artery in left ventricle. *A*, frankly fibrinoid collagen, bordered by granuloma cells, in adventitia and paraadventitia; panarteritis nodosa absent. Weigert-hematoxylin and eosin. × 743.

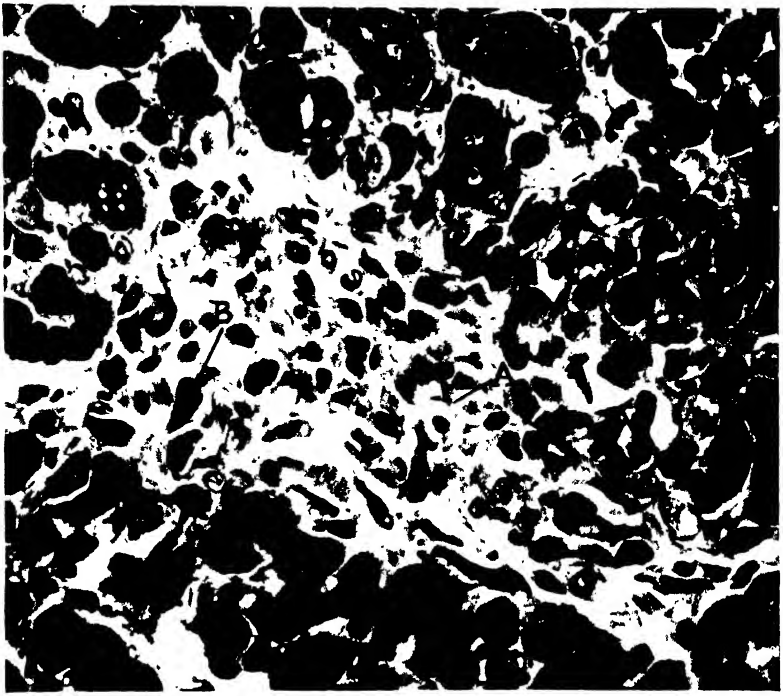
FIG. 6. Rabbit 71-77,—adventitial and paraadventitial coronal granuloma in interventricular septum. *A*, center of focus of frankly fibrinoid collagen; *B* and *C*, indistinct cell masses; *D*, cell with 3 nuclei; *E*, cell with fibrocytoid nucleus; *F*, cell with owl-eyed nucleus; many cells have indistinct cytoplasmic outlines; panarteritis nodosa absent. Hematoxylin and eosin. × 861.

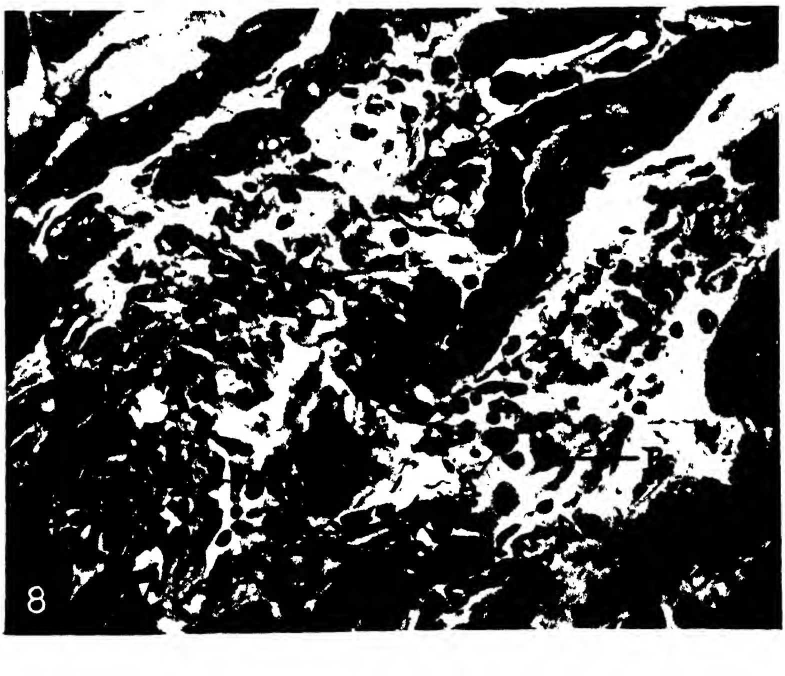
(Murphy and Swift: Induction of cardiac lesions)

PLATE 40

FIG. 7. Rabbit 71-80 (see Table III), sacrificed 10 days after last of 6 infections; autopsy blood cultures negative; reticular myocardial granuloma in interventricular septum. Cells interspersed in interlacing network of (A) swollen collagen fibers; *B*, cell with abundant basophilic cytoplasm, vacuolation of nuclei and cytoplasm of adjacent myofibers. Giemsa stain × 621.

FIG. 8. Rabbit 71-80, two mosaic myocardial granulomata in left ventricle; granuloma cells lodged between collagen masses. *A*, cell with 3 nuclei; *B*, cell with pyknotic nucleus and abundant raggedly outlined cytoplasm; most cells have indistinct cytoplasmic outlines; disintegration of adjacent myofibers. Hematoxylin and eosin. × 404.



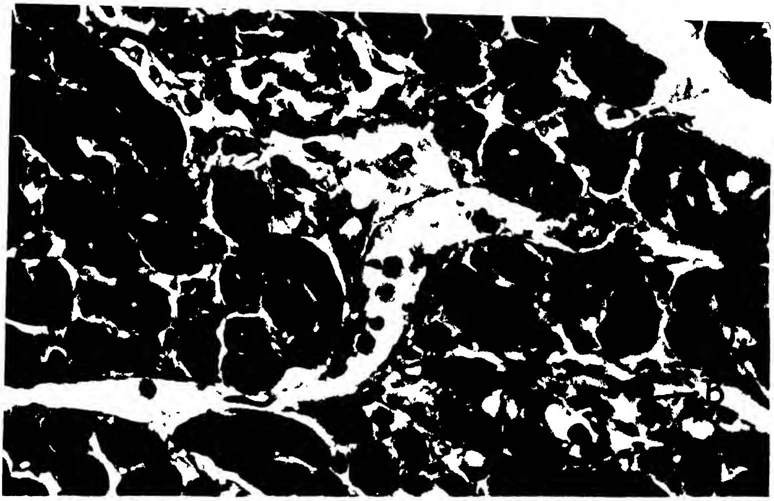


8

PLATE 41

FIG. 9. Rabbit 71-80 (see Table III), left ventricle; *A* and *B*, 2 mosaic myocardial granulomata; foci of frankly fibrinoid collagen in granuloma *A*. Hematoxylin and eosin. $\times$ 465.

FIG. 10. Rabbit 71-77, endocardial nodule on chorda tendineae at mitral leaflet attachment. Numerous multinucleated cells surrounding *A*; many cells with basophilic cytoplasmic streamers surrounding *B*; interstitial inflammation, *C*. Giemsa. $\times$ 255.

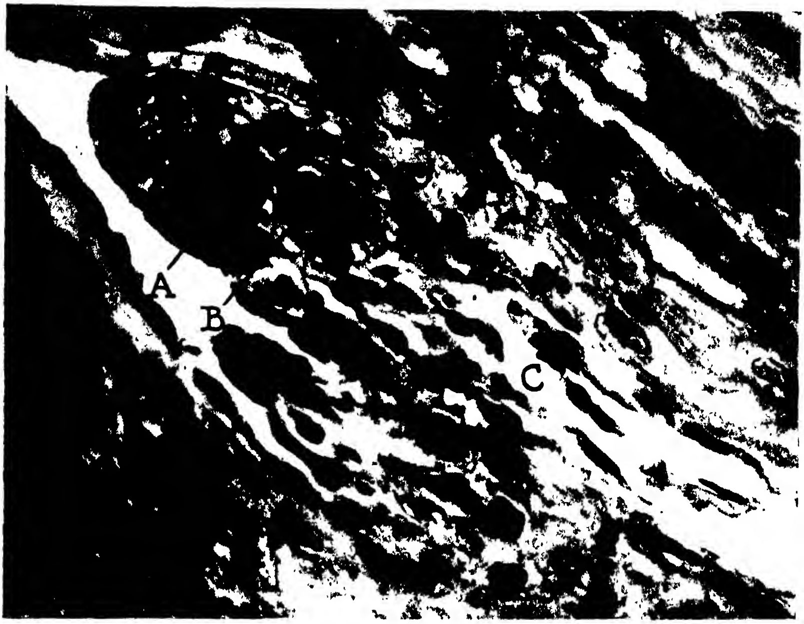


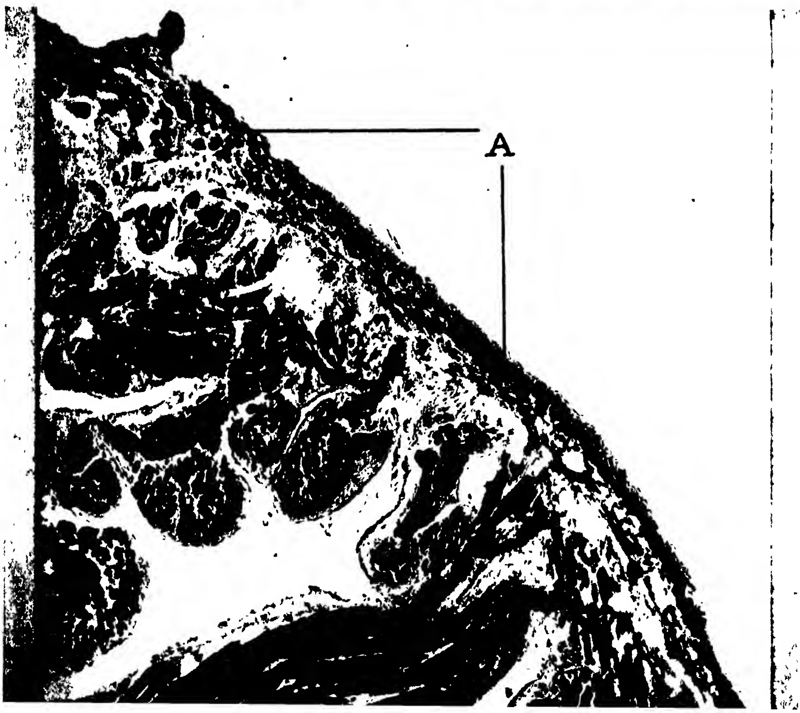
(Murphy and Swift: Induction of cardiac lesions)

PLATE 42

FIG. 11. Rabbit 70 58 (see Table I), died 13 days after last of 8 infections, negative blood cultures 2 days prior to and at autopsy. Left ventricle; mosaic nodular granuloma arising from thin walled vein; granuloma cells lodged between frankly fibrinoid masses (*A*); cell at *B* has 2 nuclei; several cells with owl-eyed nuclei; axially arranged cells surrounding *C* have streamers of cytoplasm; dissolution of adjacent myofibers. Masson trichrome stain. × 659.

FIG. 12. Rabbit 70 71, sacrificed 16 days after last of 8 infections. Left auricle; *A*, epi and subepicardial collagen converted into frankly fibrinoid material; Mallory aniline blue stain. × 127.


A
B
C


A

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, June, 1949,
Vol. 179, No. 2, pp. 743–756]

PHOTOMETRIC MEASUREMENT OF PLASMA pH

BY DONALD D. VAN SLYKE, JAMES R. WEISIGER, AND K. KELLER VAN SLYKE*

(*From the Hospital of The Rockefeller Institute for Medical Research*)

(Received for publication, December 30, 1948)

The advantages of the spectrophotometric technique in applications of indicator methods were pointed out in 1924 by Brode (1) and by Holmes (2, 3) who emphasized the accuracy of readings, the freedom from error from phenomena of dichromatism, the ease with which corrections can be made for color or turbidity in the material examined, and the small amounts of fluid that can be used. Over comparator technique the photometric has also the advantage that the latter does not require the continuous use of numerous standards.

Nevertheless the spectrophotometer has not found general application in measurement of plasma pH. Perhaps one reason is that the careful studies of Robinson and Hogden (4) on the optical density curves of phenol red in serum, buffered to known pH levels by mixing with phosphate and veronal buffers, showed peculiar effects of the plasma proteins on the density curves of the dye. The wave-length at which the dye showed maximal optical density was shifted from its normal value of 560 mμ, and dye densities measured at 560 mμ were significantly decreased by the presence of the proteins. The effect was greater when phosphate was used than when veronal buffers were employed.

Results in the present paper show that when human plasma is diluted with 20 volumes of neutral 0.9 per cent NaCl solution containing phenol red, the only buffers present being those of the plasma, effects of the proteins on the optical density of the dye, such as those observed by Robinson and Hogden in serum diluted with phosphate or veronal buffer solution, either do not occur, or occur only to such an extent as to balance a slight increase in pH caused by diluting the plasma with the NaCl solution. Consequently it is possible to estimate human plasma pH from the optical density of phenol red in the diluted plasma with a standard deviation, from the pH determined in undiluted plasma by the hydrogen electrode, of only $\pm$0.02 pH unit.

The indicators, temperature control, and conditions for diluting the plasma are the same as those used by Hastings and Sendroy (5) in colorimetric plasma pH measurements by means of test-tube comparators, but changes in so many details have been found expedient in adapting the procedure to the spectrophotometer that their description appears desirable.

* Present address, Medical College Hospital, Richmond, Virginia.

Relation of Optical Density to pH and pK′ of Indicator

With the sulfonphthalein indicators (4–8) it has been found that the relation of optical density to pH can be formulated, for the useful ranges of the indicators, on the assumptions (1) that the indicator is divided into the undissociated acid, HA, and the anion, A^-, according to the Henderson-Hasselbalch equation for weak acids, the anion A^- representing the dissociated alkali salt of the indicator, and (2) that each of the two forms of the indicator has its own characteristic curve of optical density *versus* wavelength of transmitted light, the densities of the two forms being additive.

Let E_1 and E_2 be the optical densities of the alkaline and acid forms of the indicator, respectively, measured in solutions of unit concentration (*e.g.*, 1 mg. or 1 mM per liter), and with a transmitting layer 1 cm. in length. Let D be the measured density at any given pH in the useful range of the indicator, in a solution of S concentration of total dye, with a transmitting layer of l cm. Let f be the fraction of the indicator in the alkaline form, A^-, and $1-f$ be the fraction in the acid form, HA. Then, *for light of a given wave-length,*

$$D = lE_1 f S + lE_2(1-f)S \tag{1}$$

When D_1 and D_2 are the optical densities of the alkaline and acid forms, respectively, of the indicator measured in a cuvette presenting the same l used to measure D, and in a solution of the same concentration, S, used to measure D, the equation simplifies to

$$D = D_1 f + D_2(1-f) \tag{2}$$

The Henderson-Hasselbalch equation, $\text{pH} = \text{pK}' + \log([A^-]/[HA])$ may be written as $\text{pH} = \text{pK}' + \log(f/(1-f))$. From Equation 2, $f = (D - D_2)/(D_1 - D_2)$. Whence $(f/(1-f)) = (D - D_2)/(D_1 - D)$, and

$$\text{pH} = \text{pK}' + \log\frac{D - D_2}{D_1 - D} \tag{3}$$

When, at the wave-length used, $D_2 = 0$ (*e.g.*, phenol red with wave-length 550 to 560 mμ (4)), Equation 3 simplifies to

$$\text{pH} = \text{pK}' + \log\frac{D}{D_1 - D} \tag{4}$$

The above formulation applies to the method described in the present paper.

In the application of Equations 3 and 4 to pH measurement, requirements for accuracy are that the measurements of D be made with solutions of the same dye concentration, S, and in cuvettes presenting the same l,

used in determining the constants D_1 and D_2; accurately calibrated cuvettes and accurately measured additions of dye are therefore essential.

With some indicators the necessity for constant l and S can be avoided by measuring in the same cuvette two optical densities, D_a and D_b, at two different wave-lengths, a and b, such that the pH is indicated by the ratio $D_a:D_b$. The theoretical basis of the procedure may be formulated from Equation 1 as follows, subscripts a and b being used to indicate values at the wave-lengths a and b respectively.

$$\frac{D_a}{D_b} = \frac{Sl[fE_{a1} + (1 - f)E_{a2}]}{Sl[fE_{b1} + (1 - f)E_{b2}]} \tag{5}$$

The values of S and l cancel out, and the ratio $D_a:D_b$ becomes the measure of f and hence of pH. The curve of $D_a:D_b$ *versus* pH can be plotted by calculating f for values of pH calculated by the Henderson-Hasselbalch equation in the form, $f = 1/(1 + \text{antilog }(\text{pK}' - \text{pH}))$.

Freedom from necessity for accurate addition of dye to obtain a constant S value may offer a significant advantage when small volumes are used. On the other hand, the ratio procedure has the disadvantage that it requires two readings, so that their errors are additive. The procedure has recently been used by Rutledge (7) for plasma pH determinations with phenol red, with measurement of densities at the wave-lengths 565 and 420 mμ, at which maximal densities are approached by the alkaline and acid forms of the indicator, respectively.

Special Apparatus

A photometer, preferably capable of receiving cuvettes in the form of test-tubes.[1]

A small water bath set at 38.5° and provided with a rack to hold six or more cuvettes.

Cuvettes, cylindrical, of about 16 mm. bore for macrodetermination, and about 10 mm. for microdetermination.[2]

[1] The present work was done chiefly with the Coleman junior spectrophotometer, Coleman Electric Company, Maywood, Illinois.

[2] As cuvettes we have used "culture" test-tubes, No. 9446 of the Arthur Thomas Company, 150 × 18 mm. external measurement (16 mm. bore) for macrodeterminations, 75 × 12 mm. (10 mm. bore) for microdetermination. The cuvettes of each set were tested for uniformity by measuring in them the optical density of a solution of phenol red, 2 mg. per liter, in 0.002 N NaOH, with light of 550 mμ. The solution showed an optical density of about 0.40 in the larger cuvettes and 0.23 in the micro cuvettes. A set of each size is selected such that each tube of a set gives an optical density within ±0.5 per cent of the mean and shows no change when the tube is rotated. From 100 tubes it is usually possible to select a set of twenty which meet these requirements. We have found that this selection yields more uniform cuvettes and is more economical than the purchase of calibrated cuvettes.

A 2 ml. graduated pipette drawn out near the tip into a capillary about 30 mm. in length and 1 to 1.5 mm. in bore. The capillary is used for delivering plasma into saline-dye solution under a layer of oil.

Permanent Solutions

Stock Solutions of 0.5 M Na_2HPO_4 (142.0 Gm. per Liter) and KH_2PO_4 (136.1 Gm. per Liter)—Anhydrous salts are used. The solutions are kept in Pyrex flasks in an ice box. The stock solutions are made of 0.5 M instead of M/15 concentration because the latter are less stable. From the Na_2HPO_4 solution crystals separate in the cold, but they are readily redissolved by warming each time the solution is used. The solutions in stoppered Pyrex flasks keep unchanged in the refrigerator for at least 1 year. The Na_2HPO_4 solution will absorb atmospheric CO_2 and change its pH value if much exposed to air. Hence the stoppers are removed from the flasks only for the intervals necessary to withdraw solution for use.

TABLE I

Preparation of Standard 0.5 M Phosphate Solutions

0.5 M stock solution		pH obtained at temperature indicated when mixed 0.5 M phosphate is diluted to M/15			
Na_2NPO_4	KH_2PO_4	20°	26°	32°	38°
ml.	*ml.*				
50.0	23.68	7.13	7.12	7.11	7.10
50.0	11.48	7.43	7.42	7.41	7.40
50.0	5.57	7.73	7.72	7.71	7.70

0.5 M Mixed Phosphate Solutions—These three solutions are made by mixing the above stock solutions in the proportions shown in Table I. Into each of three Pyrex Erlenmeyer flasks of 125 ml. capacity pipette 50 ml. of the 0.5 M Na_2HPO_4 solution. Then from a 25 ml. burette measure the volumes of 0.5 M KH_2PO_4 solution indicated in Table I. The solutions will keep unchanged for a year if kept stoppered in a refrigerator. They are used for preparing the less stable M/15 standard phosphate solutions.

The proportions of Na_2HPO_4 and KH_2PO_4 for different pH values are interpolated from the data of Hastings and Sendroy (5) who determined with the hydrogen electrode at 20° and 38° the pH values of twenty-five mixtures of M/15 phosphates over the pH range from 6.8 to 8.1, their electrodes being standardized with 0.1 N HCl, which was assumed to have a pH of 1.08 at both temperatures.[3] Because of the

[3] The reasons for the utility of 0.1 N HCl, with assumed pH of 1.08, as a standard have been presented by Cullen, Keeler, and Robinson (9), on grounds that appear to be still valid. This standard is the present basis of many of the data used in biochemistry, such as the values of the pK′ of the Henderson-Hasselbalch equation used

small and linear change of the pK′ of phosphate with pH, the simplest procedure to obtain exact interpolation, and to detect deviations of individual data from the trend of the series, is to plot the best line of the equation, $pK' = a + b\ pH$, and estimate values of pK′ for desired pH values by interpolation on this line. The best lines for 20° and 38° are indicated by the equations

$$pK'_{20°} = 7.125 - 0.045\ pH_{20°} \tag{6}$$

$$pK'_{38°} = 7.107 - 0.0467\ pH_{38°} \tag{7}$$

Of the 50 sets of Hastings and Sendroy's values, all but two at 20° and one at 38° gave values for pK′ within ±0.004 unit of those indicated by Equations 5 and 6.

From these equations R, the ratio $Na_2HPO_4:KH_2PO_4$, of the Henderson-Hasselbalch equation, $pH = pK' + \log R$, is defined for M/15 phosphate solutions at the two temperatures by Equations 8 and 9.

$$\log R_{20°} = 1.0450\ pH_{20°} - 7.125 \tag{8}$$

$$\log R_{38°} = 1.0467\ pH_{38°} - 7.107 \tag{9}$$

When 50 ml. of Na_2HPO_4 solution are used, the ml. of KH_2PO_4 are calculated for any pH as $50/R$.

Phenol Red, Stock 0.1 Per Cent Solution—Grind 100 mg. of phenol red with 28.2 ml. of 0.01 N NaOH till dissolved, and dilute to 100 ml. (6). For several days after the solution is prepared the phenol red may undergo change, indicated by a shifting of the pH-optical density curve prepared with solutions of the dye in phosphate buffers. Hence, in order to have a stock solution from which working solutions of relatively constant optical properties can be prepared, it is preferable to prepare the stock solution several days before use of it is begun.

Phenol Red Solution, 80 Mg. per Liter—Dilute 20 ml. of 0.1 per cent phenol red solution to 250 ml. with water. Store in a Pyrex flask in the refrigerator. It will usually keep several months without change in optical density values. The stability of the dye is checked by repeating the determination of the standard curve of pH *versus* optical density (see below) once a month or oftener.

NaCl Solution, 22.5 Per Cent (225 Gm. per Liter).

---

for bicarbonate solutions (10), for blood plasma (9, 11, 12), and for urine (13). pH values obtained on the basis of this standard agree nearly within the limit of experimental error with values at 18° based on the original phosphate buffer solution of Sørensen (14). 0.1 N HCl was used as standard by Sendroy, Shedlovsky, and Belcher (15) together with the acetate standards of MacInnes, Belcher, and Shedlovsky (16). Liquid junction potential made the 0.1 N HCl give E_0 values less accurately reproducible than values by the acetate standards in the glass electrode. In the Clark type of hydrogen electrode, however, the HCl values were found reproducible, and, in agreement with the acetate values, to less than 0.01 pH (MacInnes, personal communication).

NaCl Solution, 0.9 Per Cent—Dilute 10 ml. of the 22.5 per cent solution to 250 ml.

Neutralized Mineral Oil—In a 500 ml. separatory funnel place about 200 ml. of mineral oil, an equal volume of water, and a few drops of 0.1 per cent phenol red solution. Add 0.02 N NaOH a drop at a time with vigorous shaking until the water solution of indicator becomes permanently pink. Centrifuge the oil to get out suspended water droplets. Decant the clear oil with care that none of the water from the bottoms of the centrifuge tubes is mixed with the oil. Store in a stoppered flask.

Approximately 0.02 N NaOH, kept in a Pyrex vessel and protected from exposure to atmospheric CO_2.

Solutions Made Immediately before Use

Neutralized Saline-Dye Solution with 8 Mg. of Phenol Red per Liter—This solution is prepared immediately before it is to be used, as the pH is likely to fall too much if the solution stands for more than an hour or two. In a 100 ml. volumetric flask place 4 ml. of the 22.5 per cent NaCl solution and about 80 ml. of water, and measure in accurately 10.00 ml. of the 80 mg. per liter phenol red solution. Add by drops 0.02 N NaOH solution, stirring by rotating the flask after each addition, until the color of the solution, judged by the eye, indicates that the pH is approaching 7.4. Then fill the flask to within about 0.2 ml. of the mark, and continue addition of the 0.02 N NaOH a drop at a time until the pH at room temperature is between 7.4 and 7.6. The pH is tested at this stage after each addition of NaOH by pouring about 10 ml. of the solution into a cuvette and reading quickly the optical density. The solution is then returned to the 100 ml. flask, and, if necessary, more 0.02 N NaOH is added. This procedure is repeated until the pH is raised to between 7.4 and 7.6. It is better to have it a little above 7.4 rather than below, because the pH tends to fall from absorption of atmospheric CO_2 while the solution is being used. If the pH falls below 7.4 before a series is finished, it may be restored by adding a drop of 0.02 N NaOH to the unused residue of the solution. Keep the 100 ml. flask containing the solution stoppered to retard absorption of atmospheric CO_2. As shown by Hastings and Sendroy (5), the accuracy of the colorimetric pH determination is not significantly affected if the pH of the saline dye solution is within ±0.2 pH unit of the plasma pH.

M/15 Standard Phosphate-Dye Solutions of pH 7.10, 7.40, and 7.70 (at 38°) Containing 7.62 Mg. of Phenol Red per Liter—(These are needed only when the pH-optical density curve is checked.) For standards for the macro-procedure described below, prepare six dry test-tube cuvettes with stoppers. Duplicate cuvettes are prepared with each of the three phosphate solutions of Table I. Into each cuvette measure 8.15 ml. of water, 1.35 ml. of 0.5 M

mixed phosphate solution (Table I), and, from a calibrated pipette, 1.000 ml. of the 80 mg. per liter phenol red solution, making a total volume of 10.5 ml. After adding the dye solution, mix the contents of each cuvette with a footed rod and at once stopper the cuvette to protect from atmospheric CO_2.

If the microprocedure is to be used, one solution of each pH is prepared as above, and duplicate portions of each are pipetted into micro cuvettes.

The solutions with the three pH values are used on the same day on which they are made.

Preparation of Standard pH-Optical Density Curves

Prepare three pairs of cuvettes with M/15 phosphate of $pH_{38°}$ 7.10, 7.40, and 7.70, respectively, as described above.

Two curves are prepared, one with the standard phosphate-dye solutions at room temperature, the other with the same solutions warmed to 38°. The six tubes provide duplicate readings for each pH point.

For the room temperature curve the optical densities of the freshly prepared standard phosphate-dye solutions are read in the photometer, the zero point being set with a water blank before each reading. (This curve is used only for checking the approximate pH of saline-dye solutions.)

For the 38° curve the six cuvettes containing the standard phosphate-dye solutions are placed in a bath of water of temperature 38.5°. The cuvettes are immersed so that the surfaces of their solutions are below the level of the water surface in the bath. 5 minutes in the bath suffice to bring the solutions to its temperature. Each tube is removed from the bath, wiped, and its optical density is quickly read with a wave-length of 550 mμ before its temperature falls below 38°. (1° temperature change alters the apparent pH by about 0.01 unit.) The zero point of the photometer is set before each reading with a water blank, which need not be warmed.

The densities for the 38° curve are plotted for the pH values 7.10, 7.40, and 7.70; the densities for room temperature are plotted for the pH values indicated by Table I. Since the curves are nearly linear, three points suffice for each. They are plotted on a scale which permits estimating densities to ±0.001 and pH to ±0.002. Fig. 1 shows an example of the curve obtained with cuvettes of approximately 16 mm. bore.[2]

MACROPROCEDURE FOR DETERMINATION OF PLASMA PH

Preparation of Duplicate Saline Plasma-Dye Solutions and of Blank Solution—Draw about 5 ml. of blood without exposure to air and run the blood under oil previously placed in a tube that contains a dry film of heparin

(0.2 mg. per ml. of blood) or of neutral potassium oxalate (1 to 2 mg. per ml. of blood).

Centrifuge under conditions avoiding loss of CO_2. Blood centrifuged under a thick layer of oil may lose enough CO_2 to the oil to raise appreciably

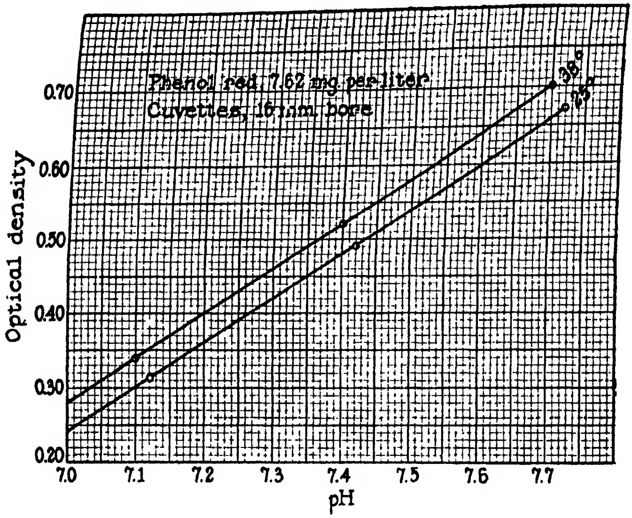


FIG. 1. Type of nearly linear curve of optical density of phenol red, measured with light of 550 mμ wave-length, at pH 7.1 to 7.7.

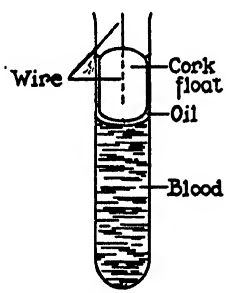


FIG. 2. Test-tube cuvette with cork float, oil, and blood, prepared for anaerobic centrifugation. See foot-note 4.

the pH of the plasma. A convenient device for anaerobic centrifugation is the float shown in Fig. 2.[4] The float is inserted after the blood has been

[4] The float is of a diameter about 1 mm. less than the bore of the centrifuge tube. The float may be made of thin glass tubing, a flattened nob being left at the top to be grasped by pincers when the float is withdrawn from the tube. The float may also be made of cork, by grinding a stopper to a cylinder of the necessary diameter, rounded at both ends as shown in Fig. 2. To facilitate withdrawing the cork from tubes, a thin copper wire is forced part way into the cork, as shown in Fig. 2.

run under the oil and mixed with the anticoagulant. Then, by gently pressing a capillary tube attached to suction against the upper edge of the float, oil is withdrawn until the bottom of the float sinks nearly to the surface of the blood, leaving a layer of oil only 1 or 2 mm. thick between float and blood. The tube is centrifuged with the float in place. Just before plasma is withdrawn for the pH determination, the float is removed from the tube, leaving a thin layer of oil, which protects the blood during the time required for pipetting out the plasma sample.[5]

Pipette two 10.00 ml. portions of the neutralized saline-dye solution into two cuvettes, for duplicate determinations, and immediately cover with neutralized mineral oil. In a third cuvette place 10 ml. of 0.9 per cent NaCl solution without dye or oil to receive plasma for the plasma blank.

Draw 1.6 ml. or more of plasma into the 2 ml. graduated pipette, the tip of which is drawn out into a capillary (see "Special apparatus").

Run 0.5 ml. of plasma into each of the two cuvettes containing 10.00 ml. of saline-dye solution under oil, the drawn out tip of the pipette being thrust through the oil into the saline-dye solution during the delivery.

A third 0.5 ml. portion of plasma, to serve as a blank, is then delivered into the cuvette that contains 10 ml. of 0.9 per cent NaCl without dye or oil.

Mix the solutions in all three cuvettes by stirring with clean, footed rods.

Place all the cuvettes in a water bath at 38.5°. Begin readings after the cuvettes have been in the bath 5 minutes.

The use of duplicate solutions of plasma plus dye adds security against error from contaminated or inaccurately calibrated cuvettes. The readings of the duplicates seldom differ by more than 0.01 pH, however, and the duplicate plasma solution may be omitted when plasma for it is lacking.

Photometric Readings—The optical density, D, of the dye in the dye-plasma solution is read, with the dye-free plasma-saline solution as a blank. Readings are made with the solutions at 38°, as described for readings of the phosphate-dye solutions in preparing the standard curve.

In a photometer in which the blank and unknown are read in succession with the cuvettes in the same light beam, it is convenient, for a series of readings, to set the zero point with a water blank, and read the densities of both the plasma-saline blank and the plasma-dye solution, rather than

[5] In a recent paper Rosenthal (17) has found that in order to obtain pH values for plasma exactly equal to those of the blood as drawn it is necessary to centrifuge at 38°. If the blood was cooled to 20° and centrifuged at that temperature, and the separated plasma was then warmed back to 38° for pH measurement, the pH obtained was about 0.03 unit greater than that observed if the centrifugation was carried out at 38°. However, practically all the data in the literature on the pH of blood plasma under physiological and pathological conditions have been obtained from plasma centrifuged at room temperature, and, for purposes of comparison with these data, it appears justifiable to continue the practice of centrifuging at room temperature.

to reset the zero of the photometer with each plasma blank. Then the dye density, D, is calculated as $D = D_1 - D_2$, where D_1 is the density of the plasma-dye solution and D_2 is the density of the plasma blank. The setting of the zero with the water blank is checked with each plasma reading.

Calculation

The pH is estimated by interpolation of D on the standard curve of pH *versus* D at 38°.

Blank for Optical Density of Plasma—The D_2 values obtained with normal, apparently clear plasmas in the cuvettes of 16 mm. bore have varied between 0.01 and 0.04 optical density units, sufficient to affect the estimated pH by 0.015 to 0.06. In such plasmas, from normal blood not lipemic from recent meals, one could, in case of necessity from lack of material, omit the D_2 reading and subtract 0.04 pH unit from the pH estimated from the D_1 reading, with a resultant error not exceeding ±0.02 pH unit. After high fat meals have produced dense lipemia, however, D_2 values as high as 0.11 have been observed in 21-fold diluted normal plasma, and some pathological lipemias gave even higher values. These did not appear to affect the accuracy of the pH determinations when the plasma blanks were handled as described for the method.

MICROPROCEDURE FOR PLASMA PH

The procedure is identical with that described above, except that cuvettes of approximately 10 mm. bore[2] are used, and one-fifth the volumes of plasma and saline-dye solution taken for the macroprocedure, the amount for each cuvette in the micromethod being 0.1 ml. of plasma and 2 ml. of the saline-dye solution.

MICROPROCEDURE WITH WHOLE BLOOD

As shown by Hawkins (18), and confirmed by Hastings and Sendroy (5), if whole blood is mixed with 10 times its volume of neutral saline-dye solution and immediately centrifuged, estimation of the pH from the color of the supernatant solution gives the same results as those obtained by first separating the plasma and then diluting it with 20 volumes of the saline-dye solution, as in the procedures described above. We have performed a few comparative analyses which indicate that similar identity of results is obtained when the measurements are based on optical densities.

The reagents are the same as for plasma, except that the dye in the saline-dye solution mixed with the blood is made 5 per cent stronger (5). Hence, instead of diluting 10 ml. of the 80 mg. per liter dye solution to 100 ml., 10.5 ml. are diluted to 100 ml. to prepare the saline-dye solution.

Procedure

0.2 ml. of whole blood is run into a micro cuvette containing 2 ml. of neutral saline-dye solution, which is covered with a few mm. of oil and mixed with the blood. The oil is replaced by low melting paraffin, and the tube is at once centrifuged. The blood blank is prepared in the same way, except that the blood is mixed with 0.9 per cent NaCl containing no dye.

The centrifuged cells should settle so closely to the bottom of the cuvette that they are below the path of light in the photometer. If this is not the case, the bottom of the slit in the adapter, in which the cuvette rests during readings, is covered with black paper to a sufficient height to insure that the cells will remain below the path of light. The photometer readings must be made within a half hour after centrifugation, or plasma pH may fall from acid formed in the cells.

EXPERIMENTAL

Determination of Plasma pH with Hydrogen Electrode

Preparation of H_2-CO_2 Mixtures—A low pressure steel tank was connected through heavy walled rubber tubing to a T-tube connected through stop-cocks to a flask of solid CO_2 and to a high vacuum pump. To remove air the tank was alternately evacuated and allowed to fill to atmospheric pressure with CO_2 three times. It was then evacuated again, and sufficient CO_2, measured by pressure, was admitted to give the desired amount in the final CO_2-H_2 mixture. The tank was then coupled to a high pressure tank of hydrogen and loaded to a total of 50 pounds positive pressure. Samples of the gas were withdrawn over mercury and analyzed for carbon dioxide content by the isolation method of Van Slyke, Sendroy, and Liu (19). Mixtures were thus prepared of the composition indicated in Table II.

Preliminary Equilibration of Plasma with H_2-CO_2 Mixture—10 ml. of fresh plasma were introduced into a 500 ml. separatory funnel fitted at the mouth with a tube closed with a pinch-clamp on rubber tubing. The funnel was evacuated with a water pump and filled with hydrogen-carbon dioxide mixture from the tank. The funnel was transferred to the 38° room and allowed to attain atmospheric pressure by quickly opening and closing the stop-cock as the gas warmed. Equilibration with the gas was obtained by rotating the funnel and passing fresh H_2-CO_2 mixture through it.

pH Measurement in Clark Cells—Four Clark (20) cells (Cullen modification (21)) were connected through saturated KCl liquid junction to a saturated KCl-calomel electrode and each was filled completely with the previously equilibrated plasma. To assure complete equilibration fresh

gas mixture was passed through each cell for 1 minute, displacing part of the plasma. The cell was then closed, except for the hydrogen inlet tube, and was rocked for 15 minutes with the hydrogen-carbon dioxide mixture at atmospheric pressure.

TABLE II

Comparison of Electrometric and Photometric pH Values at 38°

Plasma No.	Photometric, pH_P	Electrometric		$pH_H - pH_P$	CO_2 content of H_2 used to saturate plasma	Acid or alkali added to plasma
		Hydrogen electrode, pH_H	Glass electrode, pH_G			
					per cent	
1	7.60	7.58	7.60	−0.02	4.90	0
2	7.32	7.32	7.34	±0.00	4.90	0
3	7.21	7.20	7.20	−0.01	6.22	0
4	7.23	7.22	7.19	−0.01	6.22	0
5	7.24	7.24	7.23	±0.00	6.22	0
6	7.14	7.13		−0.01	6.22	0.025 vol. 0.2 N lactic
7	7.63	7.63	7.65	±0.00	1.97	0
8	7.63	7.65	7.65	+0.02	1.97	0
9	7.35	7.37		+0.02	1.97	0.025 vol. 0.2 N lactic
10	7.36	7.33		−0.03	5.25	0
11	7.43	7.47	7.47	+0.04	4.18	0
12	7.50	7.54	7.52	+0.04	4.18	0
13	7.51	7.52		+0.01	4.18	0
14	7.53	7.53		±0.00	4.18	0
15	7.44	7.46	7.43	+0.02	4.18	0
16	7.17	7.19		+0.02	4.18	0.015 vol. 1 N lactic
17	7.34	7.36		+0.02	6.65	0
18	7.65	7.64	7.63	−0.01	6.65	0.04 vol. 1 N NaOH
19	7.27	7.27	7.27	±0.00	4.48	0.015 " 1 " HCl
20	7.07	7.03	7.01	−0.04	4.48	0.025 " 1 " "
21	7.83	7.82		−0.01	4.48	0.04 " 1 " NaOH
22	6.93	6.89		−0.04	5.82	0.025 " 1 " HCl
23	7.68	7.68		±0.00	5.82	0.04 " 1 " NaOH
24	7.39	7.40	7.42	+0.01	5.82	0
25	7.16	7.18	7.17	+0.02	5.82	0.015 vol. 1 N HCl
26	7.46	7.47		+0.01	5.82	0
Average deviation.............				+0.002		
Standard "				±0.022		
Maximal "				±0.04		

Potentials were measured to the nearest 0.1 mv. by a type K Leeds and Northrup potentiometer and a sensitive Leeds and Northrup galvanometer. Usually all four cells gave similar results. If one deviated markedly from the other three, it was discarded in calculating the average.

All measurements were carried out in a room at a constant temperature of 38°. The actual temperature at the electrode was measured and the necessary corrections were applied to the pH calculation.

The platinum black electrodes were prepared with a heavy coating of platinum over gold over smooth platinum. It was generally necessary to replate after two determinations of plasma pH.

The hydrogen electrodes were standardized at 38° with each CO_2-H_2 gas mixture against 0.1000 N hydrochloric acid, the pH of which was assumed to be 1.08.[3] All measurements were corrected for actual hydrogen pressure, as calculated from the barometric pressure, the H_2 content of the H_2-CO_2 mixture, and the vapor pressure of water at 38°.

Determination of Plasma pH with Glass Electrode

After the completion of the hydrogen electrode measurements a sample of the plasma was withdrawn from a Clark cell directly into a MacInnes-Belcher (22) electrode, modified by Michaelis (23), which was fitted at the base with a stop-cock. Liquid junction to saturated KCl was established by immersing the end of the electrode in a beaker of KCl connected with a calomel half cell. The pH was determined with a vacuum tube electrometer of a type similar to that of the Cambridge Instrument Company. The entire operation was carried out in the 38° constant temperature room. The glass electrode was standardized against two known M/15 phosphate solutions, one of pH above, the other below, that of the plasma.

Determination of Plasma pH with Photometer

After the measurements with the hydrogen electrodes were completed, a 2.0 ml. graduated pipette, fitted with a bent capillary at the tip for entering the Clark cells, was filled with plasma from one of the cells. Three portions of 0.5 ml. each were run into cuvettes containing 10 ml. portions of saline-dye solution, as described for the macromethod. The fourth portion of 0.5 ml. of plasma was used for the plasma blank. The pH determinations, in triplicate, were made as described for the photometric procedure. The three values of each set usually agreed within 0.01 pH unit.

RESULTS

Comparative results by the three procedures are given in Table II. Some of the plasmas were mixed with acid or alkali, as indicated in Table II, to simulate conditions with low or high alkali reserve. In Plasmas 7, 8, and 9 the CO_2 tension was lowered, to give the conditions of a respira-

tory alkalosis in Plasmas 7 and 8, and of a compensated low alkali reserve in Plasma 9.

Both the observed maximal deviation between pH values obtained by the hydrogen electrode and the photometer, respectively, and the maximum estimated as twice the standard deviation indicate a deviation of ±0.04 as the maximum. Of the twenty-six plasmas, twenty-one showed deviations not exceeding 0.02 pH unit.

SUMMARY

The effects of plasma proteins on the optical density of phenol red, observed by Robinson and Hogden in plasma diluted with phosphate or veronal buffers, are found to be negligible in plasma diluted with 20 volumes of 0.9 per cent NaCl solution.

Procedures are described for spectrophotometric measurement of plasma pH in samples of 1.0 to 0.2 ml. of blood or plasma. The standard deviation from pH values measured by the Clark hydrogen electrode was ±0.02 pH.

BIBLIOGRAPHY

1. Brode, W. R., *J. Am. Chem. Soc.*, **46**, 581 (1924).
2. Holmes, W. C., *J. Am. Chem. Soc.*, **46**, 627 (1924).
3. Holmes, W. C., and Snyder, E. F., *J. Am. Chem. Soc.*, **47**, 221, 226 (1925).
4. Robinson, H. W., and Hogden, C. G., *J. Biol. Chem.*, **137**, 239 (1941).
5. Hastings, A. B., and Sendroy, J., Jr., *J. Biol. Chem.*, **61**, 695 (1924).
6. Clark, W. M., The determination of hydrogen ions, Baltimore, 3rd edition (1928).
7. Rutledge, R. C., *J. Lab. and Clin. Med.*, **33**, 881 (1948).
8. Sendroy, J., Jr., and Hastings, A. B., *J. Biol. Chem.*, **82**, 197 (1929).
9. Cullen, G. E., Keeler, H. R., and Robinson, H. W., *J. Biol. Chem.*, **66**, 301 (1925).
10. Hastings, A. B., and Sendroy, J., Jr., *J. Biol. Chem.*, **65**, 445 (1925).
11. Hastings, A. B., Sendroy, J., Jr., and Van Slyke, D. D., *J. Biol. Chem.*, **79**, 183 (1928).
12. Robinson, H. W., Price, J. W., and Cullen, G. E., *J. Biol. Chem.*, **106**, 7 (1934).
13. Sendroy, J., Jr., Seelig, S., and Van Slyke, D. D., *J. Biol. Chem.*, **106**, 463 (1934).
14. Sørensen, S. P. L., *Ergebn. Physiol.*, **12**, 393 (1912).
15. Sendroy, J., Jr., Shedlovsky, T., and Belcher, D., *J. Biol. Chem.*, **115**, 529 (1936).
16. MacInnes, D. A., Belcher, D., and Shedlovsky, T., *J. Am. Chem. Soc.*, **60**, 1094 (1938).
17. Rosenthal, T. B., *J. Biol. Chem.*, **173**, 25 (1948).
18. Hawkins, J. A., *J. Biol. Chem.*, **57**, 493 (1923).
19. Van Slyke, D. D., Sendroy, J., Jr., and Liu, S. H., *J. Biol. Chem.*, **95**, 531 (1932).
20. Clark, W. M., *J. Biol. Chem.*, **23**, 475 (1915).
21. Cullen, G. E., *J. Biol. Chem.*, **52**, 521 (1922).
22. MacInnes, D. A., and Belcher, D., *Ind. and Eng. Chem., Anal. Ed.*, **5**, 199 (1933).
23. Michaelis, L., *Science*, **83**, 213 (1936).

[Reprinted from The Journal of Biological Chemistry, June, 1949, Vol. 179, No. 2, pp. 757-761]

PHOTOMETRIC MEASUREMENT OF URINE pH

By DONALD D. VAN SLYKE, JAMES R. WEISIGER, and K. KELLER VAN SLYKE*

(*From the Hospital of The Rockefeller Institute for Medical Research*)

(Received for publication, December 30, 1948)

The principles of photometric pH determination have been discussed in the preceding paper (1). The conditions used for urine are essentially those devised for the visual colorimetric method of Hastings, Sendroy, and Robson (2), except that (1) the Duboscq type colorimeter is replaced by a spectrophotometer, (2) lower dye concentrations are used in the cases of brom-cresol purple and brom-cresol green, and (3) for buffer standards over the pH range from 4.9 to 5.8 acetate solutions of 0.05 M concentration are used, prepared from the pK′ values of MacInnes, Belcher, and Shedlovsky (3), instead of the 0.2 M acetate standards of Walpole (4). In comparing the optical densities of solutions of brom-cresol purple in phosphate and acetate buffers of pH 5.8, at which the useful pH ranges of acetate and phosphate buffers overlap, it was found that when 0.2 M acetate was used the density of the dye in the acetate exceeded the density in M/15 phosphate by an amount corresponding to a difference of 0.04 pH. The difference is presumably attributable to the effect on the dye of the greater ionic strength of the 0.2 M acetate. When acetate of 0.05 M concentration was used, the dye in it showed the same optical density as in M/15 phosphate of the same pH.

Permanent Solutions

Stock 0.5 M Na_2HPO_4 and KHP_2O_4—These are prepared and kept as described for plasma pH determinations (1).

Stock 0.5 M Sodium Acetate and Acetic Acid Solutions—The concentration of the acetic acid is checked by titration against standard NaOH solution. The sodium acetate solution may be prepared from $NaC_2H_3O_2 \cdot 3H_2O$ (68.03 gm. to a liter) of tested Na content, or it may be prepared by mixing equal volumes of 1 N NaOH and 1 N acetic acid that have been shown by titration against each other to be of equal normality.

Indicator Solutions—Solutions containing 1 mg. of dye per ml. are prepared by adding to 100 mg. of each dye the volume of 0.01 N NaOH indicated by Table I and diluting to 100 ml. From these stock solutions portions of 6 and 8 ml. respectively are diluted to 100 ml. to make solutions containing 60 and 80 mg. of dye per liter.

All the permanent solutions are kept in a refrigerator when not in use.

* Present address, Medical College Hospital, Richmond, Virginia.

Solutions Prepared Fresh before Using

M/15 Phosphate Buffer Solutions—These solutions are prepared from the 0.5 M solutions as indicated by Table II. The proportions of Na_2HPO_4 and KH_2PO_4 are calculated from the pK′ values of Equation 7 of the preceding paper (1).

0.05 M Acetate Buffer Solutions—These are prepared as indicated by Table III. The acetate values given in Table III are calculated from the pK′ data of MacInnes, Belcher, and Shedlovsky (3). The data of these authors show that the relation between ionic strength, μ, and pK′ of acetate buffer solutions can be expressed by the equations, $pK' = 4.769 - 0.5774\sqrt{\mu}$ at 38° and $pK' = 4.756 - 0.5774\sqrt{\mu}$ at 25°.

TABLE I

Indicators for Urine pH

Indicator	pK′ of indicator at 38° (Hastings *et al.* (2))	pH range used for urine	0.01 N NaOH added to 100 mg. indicator to make Na salt (Clark (5))	Concentration of indicator used: In solution added to 10 volumes diluted urine	Concentration of indicator used: In final urine-dye solution	Wave-length of light used	Approximate optical density of dye in cuvette of 16 mm. bore: At lowest pH of range	Approximate optical density of dye in cuvette of 16 mm. bore: At highest pH of range	Approximate mean change in density caused by change of 0.01 in pH
			ml.	*mg. per l.*	*mg. per l.*	*mμ*	*D*	*D*	*D*
Brom cresol green	4.72	4.9–5.8	14.3	80	7.27	620	0.33	0.57	0.0027
" " purple	6.09	5.8–6.9	18.5	60	5.45	590	0.28	0.71	0.0039
Phenol red	7.78	6.9–8.1	28.2	80	7.27	550	0.20	0.76	0.0046

Preparation of Standard pH-Optical Density Curves

Standard solutions are made by adding portions of 1 ml. of dye solution (60 or 80 mg. per liter) to 10 ml. portions of 0.05 M acetate or M/15 phosphate buffer solutions. The range covered by each dye is indicated by Table I. The buffer-dye solutions in test-tube cuvettes are warmed to 38° and the optical densities read as described for preparation of the optical density curves for plasma determinations (1).

The stabilities of the indicator solutions are checked once a month or oftener by repeating the density measurements with each indicator at one pH; *e.g.*, pH 5.5 for brom cresol green, pH 6.2 for brom cresol purple, pH 7.2 for phenol red. If the optical density shows a significant change, the entire curve for the indicator is redetermined.

PROCEDURE FOR DETERMINATION OF URINE PH

The urine is drawn and kept with precautions to prevent loss of CO_2, and the pH is determined as soon as possible, preferably within 2 hours.

Into uniform cuvettes, of the type described for macrodeterminations of plasma pH (1), are measured 8 ml. portions of distilled water, 2 ml. portions of urine, and 1 ml. of dye. The fluids are mixed with a footed rod, covered with neutral oil, and warmed to 38°, as described for plasma determinations (1). For each urine a blank is prepared by diluting 2 ml.

TABLE II

*M/15 Phosphate Buffers**

pH at 38°	Volumes to make 10 ml. buffer solution		Volumes when larger fraction is 25 ml.	
	Na_2HPO_4	KH_2PO_4	Na_2HPO_4	KH_2PO_4
	ml.	*ml.*	*ml.*	*ml.*
5.8	9.16	0.84	25.0	2.29
6.0	8.70	1.29	25.0	3.71
6.3	7.65	2.35	25.0	7.68
6.6	6.13	3.87	25.0	15.80
6.9	4.34	5.66	19.17	25.0
7.2	2.71	7.29	9.29	25.0
7.5	1.53	8.47	4.52	25.0
7.8	0.80	9.20	2.18	25.0
8.1	0.41	9.59	1.07	25.0

* Stock 0.5 M Na_2HPO_4 and KH_2PO_4 are diluted 7.5-fold to bring the concentration to M/15. The M/15 solutions are then at once mixed in the indicated proportions.

TABLE III

*0.05 M Acetate Buffer Solutions**

pH at 38°	$pK'_{38°}$	Volumes to make 10 ml. buffer solution		Volumes to mix when larger fraction is 25 ml.	
		Na acetate	Acetic acid	Na acetate	Acetic acid
		ml.	*ml.*	*ml.*	*ml.*
4.9	4.667	6.31	3.69	25.0	14.62
5.2	4.655	7.78	2.22	25.0	7.13
5.5	4.649	8.76	1.24	25.0	3.52
5.8	4.645	9.35	0.65	25.0	1.75

* Stock 0.5 M solutions of acetate and acetic acid are diluted 10-fold and mixed in the indicated proportions.

of urine with 9 ml. of water. The optical densities are read as described for plasma pH (1).

Since the pH of most urines will fall within the range of brom-cresol purple, a preliminary test with this dye is made. If the pH is above or below the range of brom cresol purple, the fact can usually be observed by the eye as soon as the solutions are mixed, and a fresh cuvette is prepared with phenol red or brom cresol green.

Calculation

Optical density, D, is estimated as described for plasma pH (1).

The pH of the urine diluted 5.5-fold is estimated by interpolation of D on one of the standard curves. From this pH 0.09 is subtracted as a correction for the effect of the dilution in increasing the apparent pH of the urine.

This dilution effect was noted by Hastings, Sendroy, and Robson (2) in determinations by visual comparison, and has been confirmed for photometric measurements by data in the present paper, in which the photometrically measured pH of dilute urine is compared with the pH of undiluted urine measured by the glass electrode.

TABLE IV

Comparison of pH Measured in Undiluted Urine by Glass Electrode with pH of 5.5-Fold Diluted Urine Measured by Photometer

Urine No.	pH of undiluted urine by electrode (a)	pH of 5.5-fold diluted urine by photometer (b)	Difference (b − a)
1	6.20	6.27	0.07
2	5.53	5.64	0.11
3	6.32	6.39	0.07
4	5.28	5.36	0.08
5	5.08	5.12	0.04
6	5.18	5.25	0.07
7	7.04	7.14	0.10
8	5.12	5.24	0.12
9	5.32	5.42	0.10
10	5.23	5.32	0.09
11	6.86	6.93	0.07
12	6.52	6.64	0.12
13	5.27	5.38	0.11
Mean difference			0.09
Standard deviation of difference from mean difference			±0.024

EXPERIMENTAL

Handling Urine—Consistent results for successive determinations were obtained only when the urine was kept completely protected from air. It was kept in closed vessels over mercury (tube H of Fig. 6, p. 54 of Peters and Van Slyke (6)).

Photometric Measurements—Portions of 2 ml. of urine were forced by mercury pressure from the containing vessels up into 2 ml. pipettes, from which the urine was at once delivered into the 8 ml. portions of water plus

1 ml. of dye previously measured into the cuvettes. The urine in each cuvette was at once mixed with a footed rod with the water and dye, and was covered with oil and warmed to 38° for photometric measurement, as described for plasma pH determinations (1).

Determinations by Glass Electrode—The pH of the undiluted urines was measured in Michaelis' (7) modification of the glass electrode of MacInnes and Belcher (8). The electrode was standardized with M/15 phosphate buffer solution of pH 5.80, the standardization being checked immediately before and after each urine determination. From the vessel in which the urine was kept over mercury the urine was delivered directly into the cup of the electrode, and enough was drawn down into the capillary to establish liquid junction. The unused portion of urine remaining in the cup served to protect the urine below from loss of CO_2 during the measurement. No drift in potential was apparent during the time required to make the measurements.

Results

The results of simultaneous photometric and electrometric determinations on a series of human urines are given in Table IV. They indicate that, if the correction of 0.09 given under "Calculation" is applied, the photometric values thus corrected will usually be within ±0.05 pH unit of the electrometric values.

SUMMARY

A procedure is described for photometric measurement of the pH of urine. The standard deviation from pH values determined with the glass electrode was ±0.024 pH unit.

BIBLIOGRAPHY

1. Van Slyke, D. D., Weisiger, J. R., and Van Slyke, K. K., *J. Biol. Chem.*, **179**, 743 (1949).
2. Hastings, A. B., Sendroy, J., Jr., and Robson, W., *J. Biol. Chem.*, **65**, 381 (1925).
3. MacInnes, D. A., Belcher, D., and Shedlovsky, T., *J. Am. Chem. Soc.*, **60**, 1094 (1938).
4. Walpole, G. C., *J. Chem. Soc.*, **105**, 2501 (1914).
5. Clark, W. M., The determination of hydrogen ions, Baltimore, 3rd edition (1928).
6. Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry; Methods, Baltimore (1932, 1943).
7. Michaelis, L., *Science*, **83**, 213 (1936).
8. MacInnes, D. A., and Belcher, D., *Ind. and Eng. Chem., Anal. Ed.*, **5**, 199 (1933).

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, June 1, 1949, Vol. 89, No. 6, pp. 643–668]

EFFECTS OF PROTEINURIA ON THE KIDNEY*

PROTEINURIA, RENAL ENLARGEMENT, AND RENAL INJURY CONSEQUENT ON PROTRACTED PARENTERAL ADMINISTRATION OF PROTEIN SOLUTIONS IN RATS

BY JAMES H. BAXTER, M.D.,‡ AND GEORGE C. COTZIAS, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research*)

PLATES 32 TO 34

(Received for publication, March 4, 1949)

Renal damage due to cast formation and tubular plugging by protein material, is well known in diseases associated with hemoglobinuria (1), Bence-Jones proteinuria (2), and "albuminuria" (3). The magnitude of this type of damage in Bright's disease is greatly influenced by the globulin content of the urine (4). Whether proteinuria results in renal damage other than that caused by the blockage of tubules, has not been fully determined. Cast formation may sometimes be a result of damage, as well as a cause of damage (5), and tubular lesions which did not appear to be due to obstruction have been observed with hemoglobinuria (6). However, as emphasized in Lucké's review (7), hemoglobinuria as observed clinically is often associated with shock, dehydration, and other factors, which may themselves contribute to the production of renal injury, and in diseases characterized by "albuminuria" or Bence-Jones proteinuria, one cannot always distinguish between possible effects of protein on the kidney, and other manifestations of the disease in question. The use of hemoglobin in investigations designed to determine the effects of protein itself on the kidney, is complicated by the presence of the prosthetic group.

With the advent of the treatment of nephrosis by the parenteral administration of large amounts of protein, usually with an exaggeration of the degree of proteinuria during the period of therapy, the question of the possible harmful effects of proteinuria on the kidney assumed greater importance. The same question also enters into the choice of diet in renal diseases, since the urinary protein often is increased by raising the dietary protein level.

In order to study the effects of proteinuria on the kidney, and particularly to determine whether the passage of protein through the kidney might result in renal damage other than that attributable to tubular obstruction, it was decided to attempt the production in rats of continuous proteinuria of abnormal degree, for extended as well as brief periods of time, and to follow the changes which occurred in renal morphology and composition during and following cessation of treatment, together with the changes in blood, urine, and other organs.

* A preliminary report was published in *J. Clin. Inv.*, 1948, **27**, 524.

‡ Welch Fellow in Internal Medicine of the National Research Council, now in the Department of Medicine, Johns Hopkins Hospital.

Preliminary experiments indicated that fairly continuous additions of various proteins to the blood stream, and increases in the amount of protein in the urine could be induced by twice daily intraperitoneal injections of solutions of the proteins. Efforts were made to adjust fluid and electrolyte intake, and the amount of protein administered, in such a manner that tubular obstruction would not occur in the majority of animals, and that the results would not be influenced by differences in urine volume, dehydration, electrolyte imbalance, or shock.

In addition to observing the effects of the protein injections on the kidney, some information was obtained concerning the degree and duration of retention of the various proteins in the plasma, and it was possible to make observations of a preliminary nature bearing on the questions of the mechanism of proteinuria, the passage of protein through the glomerular membranes, and the reabsorption of protein by the tubules.

EXPERIMENTAL

Materials and Methods

Animals.—Two hundred and eighty-five male albino rats of a Sherman strain were given injections as described below. The animals were approximately 24 days old when the injections were started. The maximum variation in initial weight of animals used in any series was less than 10 gm. Approximately 100 large adult rats were used as donors for rat serum which was administered to some of the experimental rats, and about 50 additional rats were employed in auxiliary experiments.

Protein Solutions.—Three per cent solutions of the following proteins, each dissolved in 0.45 per cent sodium chloride solution, were employed for injections in some of the treated groups of animals: gelatin,[1] human albumin,[2] and bovine gamma globulin.[3] Phenyl mercuric borate 0.002 per cent was present in the gelatin solution and was added to the other solutions. In addition to the experiments employing 3 per cent protein solutions, a few rats received a solution containing approximately 6 per cent gelatin in a physiological solution of sodium chloride, with 0.004 per cent phenyl mercuric borate.[1] The solutions were prepared and handled aseptically, and refrigerated when not in use.

Rat Serum.—Rat serum diluted with an equal volume of distilled water (giving approximately a 3 per cent protein solution in half-physiological salt solution), and with 0.002 per cent phenyl mercuric borate added, was used for injections in two groups of animals. The serum administered to the first group was obtained from adult rats decapitated after ether anesthesia of sufficient depth to cause respiratory depression or paralysis. When it appeared that certain undesirable effects of the serum might be due to ether retained by the serum, the serum used for

[1] Purified gelatin in isotonic solution of sodium chloride, oncotic pressure 70 mm. Hg, supplied by the Upjohn Company, Kalamazoo, Michigan, diluted with an equal volume of water. The stock solution containing approximately 6 per cent gelatin was administered without dilution to a few animals of series 9.

[2] Normal serum albumin (human) concentrated, salt-poor, E. R. Squibb and Sons, supplied by the American Red Cross.

[3] Fraction II from bovine plasma, supplied by The Armour Laboratories, Armour and Company, Chicago.

TABLE I

Summary of Experiments

	Series 1	2	3	4	5	6	7	8	9
No. of animals receiving following solutions*									
Saline	8	6	6	21		18	8	4	6
Sucrose	12								
Urea			6				8	4	
Casein hydrolysate			6	22	3		8		
Gelatin	12		6	20	3	18			12‡
Human albumin		8	6		3	12			
Bovine globulin		8	6		3	12			
Rat serum							6	4	
Total animals receiving injections (285)	32	22	36	63	12	60	30	12	18
Length of injection period, *days*	42	35	14	2	7	9	9	4	6
Time of autopsy of animals									
During course of injection period (animals)	12	6			4			4	
6 to 18 hrs. after end of injection period	6	4	30	18	8	16	30	8	6
2 wks. " " " " "						28			
2 mos. " " " " "	12	8							
At intervals of a few days after end of injection period				45		16			12
Urine collections for protein determinations									
Near end of injection period	x§	x			x	x	x	x	
1 and 2 days after end of injection period						x			
2 mos. after end of injection period	x	x				x			
Determinations of specific proteins in urine‖			x			x			
Animals and kidneys weighed at autopsy	x	x	x	x	x	x	x	x	
Determinations on blood at autopsy									
Urea	x	x	x			x		x	
Total plasma protein ($CuSO_4$ method)	x	x	x			x	x		
Plasma albumin and total nitrogen (Kjeldahl)‖						x	x		
Hemoglobin ($CuSO_4$ method)	x	x	x			x	x		
Determinations on renal cortical tissue									
Moisture content			x			x	x		
Total nitrogen (Kjeldahl)‖			x			x	x		
Tests for specific proteins‖		x	x			x			x
Kidneys examined microscopically	x	x		x	x		x	x	x

* Solutions previously described in text.

‡ Six of these animals received the more concentrated gelatin solution (see footnote 1).

§ x indicates that the examination indicated was done.

‖ These determinations were done in a limited number of cases.

the second group was obtained after decapitation, from large rats which had been placed in a nitrogen chamber until respiration stopped. These animals frequently remained in an atmosphere with little oxygen for 5 minutes or longer, and convulsions during this period were not uncommon. In both cases, the blood from the donor animals was collected with minimal contamination into tubes by means of paraffin-covered funnels and allowed to clot. The serum was separated promptly by centrifugation, placed in tubes, and immediately frozen. Portions of serum sufficient to last 24 to 36 hours were thawed and diluted as needed for injections, with constant refrigeration of the diluted serum.

Control Solutions.—One per cent urea, and 3 per cent casein hydrolysate,[4] each dissolved in 0.45 per cent sodium chloride solution, and the 0.45 per cent sodium chloride solution alone, were administered individually to control groups of animals. Phenyl mercuric borate 0.002 per cent was added as a preservative. The solutions were aseptically prepared and handled, and refrigerated between injections. In some of the later experiments, distilled water rather than half-physiological salt solution was used in the preparation of the urea and protein hydrolysate solutions in order to decrease peritoneal irritation.

General Plan of Experiments.—Comparable groups of the young rats were given, for various periods of time, twice daily intraperitoneal injections, 1 cc. per 10 gm. of body weight at each injection, of the protein, serum, and control solutions. The different series of injections, and the determinations which were done on the animals of each series are summarized in Table I. The injections were made without anesthesia, through a 21 gauge needle, care being taken to maintain aseptic conditions and to avoid injury of the liver and intestines. In order to insure an equal fluid intake and the elimination of urine of comparable volume and concentration by the animals of the different groups, *no fluid* except that administered in the injections already described was allowed. A stock diet[5] was offered *ad libitum*. For several days prior to and during the urine collections, a low residue synthetic diet[6] was substituted for the stock diet in order to decrease the bulk of the feces. Because vitamin-free injected materials were to some extent utilized in place of food by animals of some of the groups, complete vitamin supplements[7] were offered *ad libitum* to all animals. A complete salt mixture[8] was also supplied separately so that the animals could adjust their own salt intake to some extent. The animals were weighed at intervals of a few days and the amounts of injected materials were increased as indicated. Urine was collected from time to time for protein determinations. Animals from various groups were autopsied at intervals during, at the end of, and at periods following terminations of injection periods. Blood was collected for study, weight of kidneys was determined, and kidney tissue was preserved for chemical and histological study. Animals not autopsied during, or at the end of, injection periods were given water *ad libitum* beginning 24 hours after the final injection. The occasional animals which became seriously ill or died were not included in the determinations of kidney size.

Urine Collections.—Urine was collected by means of metabolic cages placed above large paraffin-coated funnels which drained into flasks containing a little toluene. Because of the

[4] Amino acids—I. C., lyophilized (acid hydrolysate of casein with added tryptophane) supplied by Biochemical Division, Interchemical Corporation, Union, New Jersey.

[5] Purina fox chow.

[6] The diet employed was the same as that described elsewhere (8), with 0.3 per cent choline chloride added. The changes in the diet did not appear to influence appreciably the kidney weight–body weight ratios or the amount of protein in the urine. The dietary treatment of protein-treated and control groups was the same in each experiment.

[7] The vitamin mixture was the same as that used in the synthetic diet (8), with added choline chloride.

[8] Osborne-Mendel salt mixture No. 2, Eimer and Amend, New York.

small size of the animals, from 3 to 6 rats were placed together in each cage. Collections were made for periods of from 12 to 24 hours. When injections were continued during the periods of urine collections the animals were not returned to the metabolic cages until leakage from peritoneal cavity, if present, had ceased. Since fairly uniform results were obtained, it was thought that subsequent leakage did not occur. Because of technical difficulties, the collections were only approximately quantitative. As indicated in Table I, urine collections were made at various times after termination of injections, as well as near the end and sometimes at earlier stages of the injections.

Urine Examinations.—The urine specimens were cleared by centrifugation, and filtered if necessary. Volume was recorded, and protein concentration determined by the Shevky-Stafford (9) or the biuret (10) method. Gelatin was precipitated by addition of tungstate or Tsuchiya's reagent after removal and estimation of other proteins by addition of an equal volume of 10 per cent trichloracetic acid. Human albumin was estimated in a few cases by determination of the turbidity produced by addition of antiserum prepared from rabbits.[9] Bovine globulin was tested for qualitatively by serological precipitation, and the relative globulin content of urines from various groups was estimated by comparing the precipitates formed by adding equal volumes of 44 per cent sodium sulfate, after adjusting pH of the urine specimens to 7.4 (11).

Urine sediment was examined microscopically in a few instances.

Blood Examinations.—At the time of autopsy, during, or sometime after termination of the injections, blood was collected into heparin-containing flasks after decapitation of the animal. Total plasma protein and hemoglobin levels were estimated from specific gravity determinations by the copper sulfate method (12). Urea was determined by the hypobromite method (13). Total plasma nitrogen and plasma albumin determinations were done in a few instances by a micro Kjeldahl method (14), using pooled plasma from the various groups.

Examination of Organs.—After comparable animals of different groups had been weighed, killed by decapitation, and thoroughly drained of blood, the peritoneal cavities were inspected for free fluid, and for evidence of peritoneal irritation, infection, or injury to organs. When fluid was present in the peritoneal cavity, the weight of the animal was corrected by deducting the weight of the free fluid. Tissues were observed for evidence of dehydration or overhydration. The size, color, and other gross features of kidneys, liver, and spleen were specifically noted. Differences in size and shade of kidneys were most impressive when the organs from comparable control and protein-injected animals were compared directly by placing the kidneys side by side. The kidneys and sometimes the liver and spleen were removed for weighing, and tissue was preserved for histological study of gross and microscopic sections, and for total nitrogen and moisture determinations.

Determination of Relative Weight of Kidneys.—The animals of the different groups were of approximately the same weight at the beginning of the injection periods, and the growth rate of all the animals was fairly uniform, at least for several weeks. In experiments which were continued for a number of weeks, there was sometimes considerable variation in weight of the animals at the time of autopsy. In the early experiments, the kidneys were removed without attached tissue, but with capsules in place, and were weighed accurately. After it was observed that some of the injected solutions produced significant thickening and increase in weight of the renal capsules, the capsules of all kidneys were removed carefully before weighing. In order that the kidneys of various animals might be compared more accurately, the ratio of kidney weight to corrected body weight was determined in each case. In each series of experiments, animals of various groups were treated similarly. In most cases, autopsies were

[9] We are indebted to Dr. Henry G. Kunkel for the determinations of human albumin in urine and renal cortical tissue.

performed about 18 hours after the last injection, with no oral fluid during the intervening period. In some of the early experiments, however, the time interval between the last injection and autopsy was less than 18 hours.

Histological Examinations of Kidneys and Other Organs.—After the kidneys were weighed they were sectioned along the median sagittal plane, and direct comparisons made of kidneys of animals of various groups. Kidney tissue was then fixed in alcohol-formol-acetic acid,[10] or sometimes in 10 per cent neutral formalin or in absolute alcohol. Paraffin sections were prepared as routine, and stained for microscopic examination with hematoxylin and eosin or with Giemsa's stain, and frequently with Mallory's connective tissue stain. A few sections from kidneys of gelatin-treated animals were fixed in absolute alcohol and stained with eosin and with several other stains in absolute alcohol, without passage through aqueous solutions. Frozen sections of formalin-fixed tissue were stained for fat in a few instances. Liver sections from some animals and sections of other organs from occasional animals also were studied microscopically.

Determinations of Water and Other Constituents of Cortical Tissue.—Cortical tissue was prepared by bisecting decapsulated kidneys and removing the medullary portion. The cortical tissue was then pressed against blotting paper to remove free fluid present in tubules and blood vessels.

Moisture determinations were made on kidneys from most of the animals. This was done by placing the cortical tissue from one-fourth to one-half of a kidney in a weighing bottle, accurately determining the wet weight of the tissue, drying in an oven at 104°C. for 6 hours, and again weighing. The percentage loss of weight by the wet tissue on drying was then calculated.

After tissue had been taken for sections and for moisture determinations, the remaining cortical tissue was placed in tubes, weighed, and frozen. Total nitrogen determinations were performed on this tissue in a few cases by a Kjeldahl method (14). In some other cases, the tissue was ground in water or saline with sand or by means of a Potter homogenizer. The solutions were cleared by high speed centrifugation, and gelatin, human albumin, and bovine globulin were precipitated as already described for the proteins in urine. Results on kidney tissue from control and protein-treated animals were compared in each instance. In a few cases, the protein injections were discontinued and the animals given water for 2 to 7 days before autopsy in order to eliminate most of the injected protein from blood and urine, small amounts of which were retained by the renal tissue. Although a few quantitative estimations of the specific protein were made, the results were of only qualitative significance because certain factors were not well controlled.

RESULTS

Most of the animals which received injections of the various substances remained well and grew fairly normally. The animals which received injections for the longer periods grew from weanlings to rats weighing approximately 200 gm. without receiving any fluids by mouth. Disturbances in health and growth of animals due to intraperitoneal infections or injuries produced at the time of injections, were rarely encountered. There was no evidence of sensitization of the protein-injected animals to the proteins, though temperatures of the injected animals were not determined following injections and sera were not examined for antibodies against the proteins. The impression was obtained that the animals which were given gelatin were less excitable and struggled less

[10] Eighty per cent alcohol 900 cc., 40 per cent formalin 50 cc., and glacial acetic acid 50 cc.

during injections than those of other groups. In the early experiments, the animals receiving protein hydrolysate appeared to be less able than animals of other groups to tolerate periods of more than 12 hours without an injection of fluid. This difference was partially removed by administering the protein hydrolysate in water rather than in sodium chloride solution, and by offering a complete salt mixture separately.

Data obtained from groups of animals injected with rat serum are included in tables and charts along with data from other groups. However, since the experiments with the homologous serum were not considered entirely satisfactory, they will be discussed separately.

Absorption of Injected Materials from Peritoneal Cavity.—The control (non-protein) solutions were absorbed somewhat more rapidly from the peritoneal cavity than were the protein solutions. In most experiments, even the protein solutions were fairly completely absorbed during the period from one injection to the next, and only in the case of one group receiving the usual 3 per cent solution of gelatin (series 4), the group receiving 6 per cent gelatin (series 9), and particularly the groups receiving rat serum did intraperitoneal accumulations of injected material present a serious problem. The animals receiving urea became dehydrated and the peritoneal surfaces did not have the normal moist appearance, though the hemoglobin levels did not appear to rise appreciably.

Changes in Plasma Proteins and Hemoglobin Levels Resulting from Injections.—Plasma protein and hemoglobin levels, which were determined in animals of the various groups at the time of autopsy of the animals, during the course of, and at intervals following the termination of injection periods, varied somewhat from group to group, and with the length of the injection period, and with the period of time elapsing between the last injection and the autopsy of the animal, but, qualitatively, the results were similar. Data obtained from animals of series 3, 6, and 7, are presented respectively in Tables II, III, and IV. Little or no change in serum protein or hemoglobin levels was observed with gelatin injections. Unfortunately studies were not done on animals of series 4 receiving the usual gelatin solution, or on those of series 9 receiving the more concentrated gelatin solution, some of which retained fluid in the peritoneal cavity, and some of which developed severe tubular damage. Albumin produced significant elevations in total plasma protein, plasma albumin, and in the ratio of albumin to globulin, with a coincident decrease in the hemoglobin level. Globulin induced an even greater rise in total plasma protein and plasma globulin, but the decrease in hemoglobin concentration was of about the same magnitude as with albumin. After termination of injections, the plasma protein and hemoglobin values returned fairly promptly toward normal.

Effect of Injections on Blood Urea Levels.—The animals receiving injections of control and protein solutions, including those of the long term experiments were found at the time of autopsy to have blood urea nitrogen values ranging between 12 and 28 mg. per cent, with the majority in the lower two-thirds of the

range, and there was no consistent difference between control and protein-treated animals. Some of the higher values were found in animals which appeared ill, but neither illness nor high urea nitrogen values were more common in protein-treated than in control animals, and neither could be attributed with any degree of certainty to specific effects of the injected materials on the kidneys. In animals given continued injections for prolonged periods and then observed for several weeks or months on a normal regime of food and water by mouth, the blood urea nitrogen values were still found to be normal. Blood urea levels were not determined in series 4 and 9 where retention of fluid in the peritoneal cavity, and renal damage, were observed in some of the animals receiving gelatin.

TABLE II

Total Plasma Protein and Hemoglobin Levels of Rats of Series 3

Injected solutions*	Total plasma protein	Hemoglobin
	gm. per cent	*gm. per cent*
Saline	6.5	10.6
Urea	6.2	9.9
Casein hydrolysate	6.5	11.0
Gelatin	6.4	10.1
Albumin	8.5	8.4
Globulin	9.5	8.3

The animals were autopsied about 6 hours after the last injection, following an injection period of 2 weeks. Each figure represents an average from specific gravity determinations on 3 rats by the $CuSO_4$ method.

* Solutions previously described in text.

Influence of Injections on Proteinuria and Urine Output.—Rapidly occurring diuresis after each injection was noted in animals receiving injections of urea, and the volume of urine excreted in 24 hour periods by these animals was usually somewhat greater than that excreted by comparable animals of other groups, despite the fact that the fluid intakes of comparable animals of all groups were equal. The rate of urine excretion was more uniform throughout the 24 hour periods in the protein-treated animals than in those receiving saline and amino acids, but the total urine volumes of animals of all these groups were approximately equal.

Urine from saline-injected animals always contained small amounts of protein, nearly always less than 1 gm. per liter, and often in the range of 0.5 gm. per liter or less. The total protein excreted per day varied with the size and age of the rat, but after an injection period of 2 weeks, starting with weanling rats, the amount was usually found to be between 1 and 2 mg. per rat per 24 hours. The amounts of protein excreted by the animals receiving amino acids

TABLE III

Plasma Protein and Hemoglobin Levels of Rats of Series 6

Injected solutions*	18 hrs. after final injection			2 days after final injection		5 days after final injection		10 days after final injection		
	Plasma protein‡	(Plasma protein) A/G§	Hb‡	Plasma protein‡	Hb‡	Plasma protein‡	Hb‡	Plasma protein‡	(Plasma protein) A/G§	Hb‡
	gm. per cent		*gm. per cent*	*gm. per cent*	*gm. per cent*	*gm. per cent*	*gm. per cent*	*gm. per cent*		*gm. per cent*
Saline	5.0	(6.0) 1.8	12.2	5.5	12.7	5.7	12.6	5.7	(5.7) 1.6	11.4
Casein hydrolysate	5.2	—	13.3	—	—	—	—	—	—	—
Gelatin	5.3	(4.8) 1.7	12.1	—	—	5.7	12.7	5.4	(5.5) 1.6	10.8
Albumin	6.5	(6.7) 3.0	10.2	5.8	11.7	—	—	5.4	(5.3) 1.6	10.9
Globulin	8.6	(9.5) 0.33	10.5	8.0	13.0	6.5	14.0	6.0	(5.9) 1.3	11.2

After an injection period of 9 days, the animals were autopsied at intervals as indicated in the table. Animals were given water *ad libitum* beginning 24 hours after the final injection.

* Solutions previously described in text.

‡ Average values calculated from specific gravity determinations on a total of 40 rats by the $CuSO_4$ method.

§ Single determinations on pooled plasma, using Howe separation and micro Kjeldahl.

TABLE IV

Total Plasma Protein and Hemoglobin Levels in Rats of Series 7

Injected solution*	Total plasma protein	Hemoglobin
	gm. per cent	*gm. per cent*
Saline	5.7	12.5
Urea	5.6	12.8
Casein hydrolysate	6.0	11.9
Rat serum	7.8‡	11.4

Animals were autopsied about 18 hours after the last injection, following an injection period of 9 days. Each figure represents an average value calculated from specific gravity determinations on 6 rats by the $CuSO_4$ method.

* Solutions previously described in text.

‡ A single determination on pooled plasma indicated that the albumin fraction was only slightly elevated.

and urea were in the same range as that of the saline animals, though the values for the urea-injected animals appeared to be consistently slightly higher than those of the other control groups, perhaps due to the diuretic effect. Additions

of sodium sulfate according to the method for globulin precipitation, to urines from the control groups, produced not more than a faint cloud, and most of the protein was presumably albumin. Urine specimens collected 1 to 2 weeks after starting injections, from the protein-treated animals, almost always contained more than 1 gm. of protein per liter of urine. The animals receiving albumin sometimes excreted as much as 5 gm. per liter or more over the 24 hour period. Globulin induced less marked elevations in urinary protein levels, while gelatin injections were usually accompanied by greater degrees of proteinuria than those observed with albumin.

The increase in urinary protein in animals injected with gelatin was due principally to the presence in the urine of gelatin; the other urinary proteins (precipitated by an equal volume of 10 per cent trichloracetic acid) did not appear to be regularly or markedly altered. Gelatin appeared in the urine and was occasionally seen in the glomerular capsules before there were appreciable changes in kidney size or visible alterations in the tubular cells. In animals injected with human albumin, preliminary determinations of the human albumin in the urine by the serological precipitin reaction suggested that only a part, probably less than one-half of the urinary protein increment was composed of the injected human albumin. The remainder was presumably rat protein, probably chiefly albumin. As in the case of the albumin, it appeared unlikely that the injected globulin was present in the urine in sufficient quantities to account for all the increase in urinary protein which accompanied injections of that protein.

In animals receiving injections of albumin and globulin, the urinary protein levels, like the plasma protein levels already discussed, remained definitely elevated for at least several days after termination of the injections. However, when urine specimens from the various groups receiving proteins were examined after periods of 2 weeks to 2 months, the urinary protein levels were found to be within the same range as in the specimens from comparable control animals.

Examination of urinary sediment from protein-treated animals revealed no abnormalities.

Gross Changes in Organs Resulting from Injections.—Examinations of the peritoneal cavities sometimes revealed adhesions between liver or intestines and omentum or adjacent organs, due to puncture wounds produced at the time of injections. Significant peritoneal infections were never recognized, if present. Prolonged injections of urea solution, and particularly of protein hydrolysate solution, appeared to produce peritoneal irritation, with thickening and opacity of peritoneal surfaces including hepatic and renal capsules. In the case of urea, the peritoneal surfaces were often less moist than with the other solutions. Saline and the 3 per cent protein solutions produced no peritoneal changes. However, the more concentrated gelatin solution used in series 9 produced definite peritoneal thickening, with rounding of the liver edges.

Occasional animals, without respect to treatment, were observed to have large dark spleens.

It was noted during the early periods of the study that the kidneys of animals which had received repeated injections of gelatin appeared enlarged and pale. The correctness of this observation was confirmed by side by side comparisons of the kidneys of control and gelatin-treated animals (Figs. 1 and 3). When sagittal sections of kidneys from gelatin-injected animals were compared with similar sections from comparable control kidneys, it was further evident that the enlargement of the kidneys from animals receiving gelatin was principally in the cortical portion. The cortical tissue appeared pale particularly through the outer two-thirds of its depth, and was separated from the pale medullary tissue by a narrow dark band at the corticomedullary junction.

In experiments employing albumin and globulin, similar but usually somewhat less marked renal enlargement was noted (Figs. 2 and 3). Paleness of some degree was seen frequently with injections of these proteins, particularly when enlargement of considerable degree occurred, but it was usually much less than with gelatin.

No gross alterations, other than the differences in size and depth of color, were noted in any of the kidneys, and the pelves and ureters always appeared normal.

There appeared to be no changes in the liver or spleen attributable to injections of protein, though these organs were accurately weighed in only a few cases. No changes were noted in the adrenal glands, which were not studied extensively.

Change in Relative Size of Kidneys Due to Injected Substances.—Since there was always some variation in size of the experimental animals at the time of autopsy, the feasibility of expressing the relative kidney size as the ratio of the weight of both kidneys divided by the weight of the animal, was investigated. It was found that this ratio was quite constant over the range of variation in animal size encountered in the individual experiments, particularly in those of not more than a few weeks' duration. This ratio, multiplied by 100, decreased steadily with growth of the animals, from approximately 1.25 in the youngest rats autopsied to about 0.67 in the rats of series 1 which were autopsied after being observed for 2 months at the end of the 6 weeks injection period.

In the early experiments, the kidneys were weighed without decapsulation. However, after the introduction of urea, amino acid, and rat serum solutions, it was observed that these substances caused sufficient thickening and increase in weight of the renal capsules to alter appreciably the kidney weight–body weight ratios, and in subsequent studies the kidneys were carefully decapsulated before weighing.

The animals receiving urea and protein hydrolysate solutions, like those receiving saline alone, exhibited little change in relative kidney size. The kidney weight–body weight ratios of the animals receiving urea were usually slightly

elevated compared with those of the other control groups, perhaps because of a decrease in body weight due to dehydration.

Protein solutions uniformly caused an increase in relative kidney size and weight. The most rapid enlargement appeared to occur in the early part of

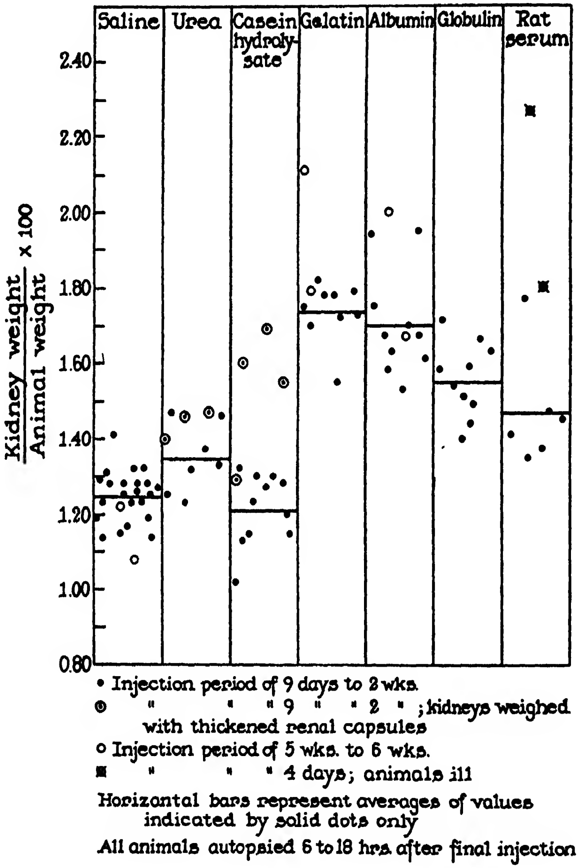


CHART 1. Relative renal size of comparable rats injected with various protein and control solutions.

the injection periods, but the enlargement was maintained as long as the injections were continued.

Gelatin caused the most rapid and usually the most marked increase in kidney size. Definite enlargement occurred within a few days, and perhaps within 1 day. Albumin ultimately caused almost as great enlargement as gelatin but the enlargement appeared to develop more slowly. Globulin produced less enlargement than gelatin or albumin.

Chart 1 shows the results of determinations of relative renal size which were made in the animals receiving injections for 9 days or longer and autopsied within 1 day after the final injection. The animals were from series 1, 2, 3, 6, and 7, with the two ill animals from series 8. Chart 2 contains further data from series 4 on the enlargement produced by short term injections of gelatin.

Reversibility of Renal Enlargement after Termination of Injections.—Chart 2 shows the changes in relative kidney size over a period of 8 days in rats of series 4, given 3 single injections of gelatin solution, compared with those of rats given injections of saline and casein hydrolysate. For some unknown reason,

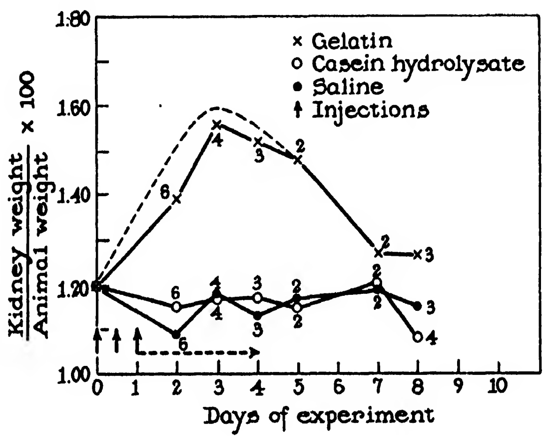


CHART 2. Showing changes in relative renal size on successive days produced by three injections of gelatin, compared with control injections. Absorption of gelatin was unusually slow as indicated by horizontal arrow, and weight of animals was not corrected for weight of peritoneal fluid. Broken line indicates approximate correction. Numerals indicate number of observations. Average weight of gelatin-treated animals autopsied on various days was: 2nd day, 46 gm., 3rd day, 48 gm., 7th and 8th days, 58 gm. Data from series 4.

possibly because the animals were autopsied at an earlier age than most, it was found that the gelatin solution had not been as completely absorbed as usual, and fluid was present in the peritoneal cavities of many of the rats for 2 or 3 days after the final injection. This continued absorption probably accounted for the further increase in relative kidney size after injections were discontinued. The chart demonstrates that a prompt increase in the relative size of the kidneys of the gelatin-treated animals occurred, followed by a prompt return toward normal as soon as the injections were discontinued and the gelatin was all absorbed from the peritoneal cavity. Evidence of some degree of renal damage was observed in the kidneys of several rats of this group, as will be discussed in more detail later.

Chart 3 shows the changes in the kidney weight–body weight ratios, during

the 10 day period following termination of a 9 day injection period, in animals receiving various protein solutions. The final injection is indicated by vertical arrow. Protein solutions had been almost completely absorbed when the first animals were autopsied on the 10th day.

The decrease in kidney weight–animal weight ratio after termination of protein injections, particularly when observations were made over a considerable period, was due in part to a dilution of the kidney weight increment which resulted from the injections, due to normal growth of the kidneys. In addition to this effect, however, there was a prompt decrease in the absolute difference

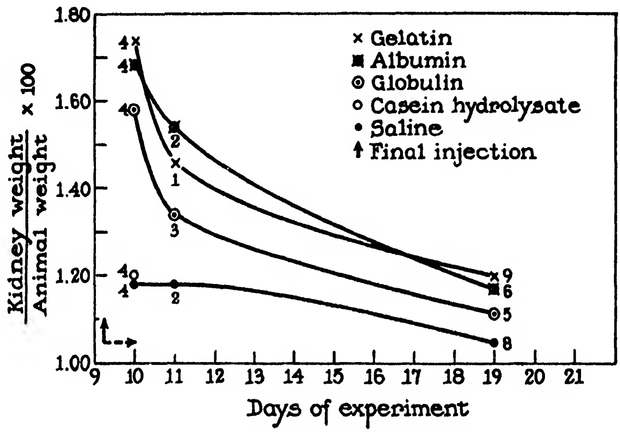


CHART 3. Showing return of kidneys of protein-treated animals toward normal size, following termination of a 9 day injection period. Data are from series 6. Numerals on chart indicate number of observations at each point. Horizontal arrow indicates duration of presence of protein solutions in peritoneal cavities of animals. Average weights of various groups of animals autopsied at different points on chart were: 10th day, 51 to 52 gm., 11th day, 52 to 56 gm., 19th day, 75 to 86 gm.

in size between the kidneys of protein-treated and control animals. Charts 2 and 3 indicate that some residual enlargements of the kidneys of the protein-treated groups probably remained at the end of the periods of observation. In series 1, there appeared to be slight enlargement of the kidneys of the gelatin-treated animals 2 months after the injections were discontinued. However, since any residual enlargement becomes a progressively smaller portion of the total renal tissue as the animals grow older, and greater variation in animal size occurs, the long term observations on small groups of animals were of doubtful significance. It can only be concluded that there was a prompt return of the kidneys of the protein-injected animals in the direction of normal size, after protein injections were discontinued.

Composition of Kidneys of Animals Receiving Various Solutions.—After it was apparent that injections of protein solutions resulted in renal enlargement,

it seemed of importance to determine whether the enlargement was due simply to an increase in the water content of the renal tissue, or whether it was due to an increase in material of approximately the same solid content as normal renal tissue. Water determinations were used to answer this question. Since the enlargement appeared to involve chiefly the cortical portion of the kidney, cortical tissue was used for the determinations. It is evident from data in Table V, representing water determinations on kidney tissue from a total of 70 animals, that only a slight part, if any, of the enlargement was due to an increase in the proportion of water to solids in the renal tissue of the protein-treated animals. The increase in weight was due to addition of water and solids in almost the same proportion present in normal kidney tissue. There was a slight increase in average relative water content of the tissue from animals

TABLE V

Moisture Content of Renal Cortical Tissue of Rats from Series 3, 6, and 7

Injected solutions*	Moisture	
	18 hrs. after final injection	10 days after final injection
	per cent	*per cent*
Saline	77.2	77.3
Urea	76.8	—
Casein hydrolysate	76.8	—
Gelatin	78.0	77.2
Albumin	78.8	78.3
Globulin	76.9	77.5
Serum	77.5	—

Injections were continued for 9 days to 2 weeks.
* Solutions previously described in text.

receiving albumin, and possibly in that of those receiving gelatin, 18 hours after termination of the injections. The 10 day values are less reliable because of the smaller number of determinations and greater spread of individual values.

Total nitrogen determinations on a few kidneys of various groups gave approximately similar results in most of the control and protein-treated animals and indicated that the protein concentration was probably normal in the protein-treated animals. The increase in kidney weight was apparently due in most instances to addition of water and protein in nearly the same ratio existing in normal kidneys.

Injected Protein in Renal Tissue.—Homogenates of renal cortical tissue from gelatin-injected animals were usually found to contain definitely more material resembling gelatin, in that it was precipitated by tungstate but not by a final concentration of 5 per cent trichloracetic acid, than was found in the renal tissue from other groups. This was true even when the determinations were made several days after gelatin injections were discontinued. However, efforts

were not made to insure quantitative extraction of gelatin or other specific proteins from the renal tissue and quantitative determinations of gelatin were not made in most cases. The desirability of further studies was evident when, during the course of the experiments, it was found that kidney tissue from *control* animals sometimes contained more than the minimal amounts of "gelatin" observed in the early experiments.

In extracts of kidneys from rats that received human albumin and bovine globulin, serological precipitin tests showed the presence of these injected proteins. The amounts of extracted human albumin were much smaller than necessary to account for the total enlargement of the kidneys associated with injections of that protein, and it was not established beyond question that the results could not be accounted for on the basis of human albumin in the blood and urine retained by the renal tissue. Quantitative determinations of the globulin were not attempted.

These results were regarded as suggestive but not conclusive evidence that the renal parenchymal cells contained quantities of the injected proteins.

Microscopic Observations.—No abnormalities or differences were noted on microscopic examination in the kidneys of animals receiving saline, (Fig. 4), urea, or protein hydrolysate.

Kidneys from animals receiving gelatin showed, in addition to the enlargement and paleness apparent on gross examination, rather marked alterations in the cells of the convoluted tubules, particularly in the outer two-thirds of the cortex (Figs. 5 and 6). The proximal tubules were probably more markedly involved than the distal segments. The cells of the convoluted tubules were enlarged and contained what appeared to be clear spaces in the cytoplasm. These clear spaces were observed in sections of tissue that had not been exposed to aqueous solutions, as well as in those subjected to the routine staining procedures. There was no increase in fat content of the cells as shown by fat stains. The lumina of the convoluted tubules appeared to be decreased in width and in some cases they were almost closed, due to encroachment by enlarged cells.

Although the kidneys of the animals receiving solutions of albumin and globulin were definitely enlarged in the gross, on microscopic examination no definite and consistent changes were noted which might have been responsible for the enlargement. There appeared to be slight cytoplasmic alterations in the cells of the convoluted tubules in some cases. Increases in size of the cells of the convoluted or other tubules, or changes in caliber of the tubular lumina, if present, were not of sufficient magnitude to be recognized, and there was no other apparent explanation for the increase in bulk of the cortical tissue. In most of the animals of the protein-treated groups, no changes suggestive of an increased production of new cells in the cortex, were observed; mitotic figures were noted in the tubules of some animals autopsied early in the course of gelatin injections.

In the kidneys of some animals receiving proteins, protein material was visible in occasional glomerular capsules and tubular lumina. Rare isolated tubules in the region of the straight segments appeared to be plugged with protein material but similar tubules also were seen from time to time, but perhaps less frequently, in control kidneys. No abnormalities were noted in glomeruli, blood vessels, or interstitial tissue, even in animals treated and observed for prolonged periods. No changes suggestive of sensitization of the animals to the injected proteins were observed.

Three or four gelatin-injected animals of series 4—the series in which absorption of the injected 3 per cent gelatin solution was unusually slow—differed further from most of the protein-treated animals in that definite evidence of renal damage was observed on microscopic examination of the kidneys. These animals in which definite damage was recognized were among those which were autopsied from 2 to 5 days after gelatin injections were discontinued. The morphological alterations were much more extensive in the kidneys of one of the animals (Figs. 7 and 8) than in those of the others. These alterations were localized principally in the inner cortical and outer medullary zones where glomerular capsules and tubules appeared dilated and contained some protein material, and the tubules were lined by flattened epithelium containing mitotic figures, large hyperchromatic nuclei, and basophilic cytoplasm. Similar dilation of glomerular capsules and tubules, not accompanied by evidences of tubular necrosis or regeneration, was present at the time of autopsy in the kidneys of some of the gelatin-treated animals of series 9, particularly those receiving the more concentrated solution. In the kidneys of the majority of animals receiving each of the 3 per cent protein solutions, no changes were observed which could be considered evidence of renal damage, unless the cytoplasmic alterations in the tubular cells induced by gelatin are interpreted as evidence of damage.

In the kidneys of animals receiving gelatin, the appearance of the cells of the convoluted tubules gradually returned toward normal after injections were discontinued, with the clear spaces decreasing in size and finally disappearing. While there was considerable individual variation, the morphological alterations largely disappeared in most cases within about a week, during which time the renal enlargement and the "gelatin" in the renal tissue also decreased.

During the periods following the injections no impressive renal cytologic changes were observed in the animals receiving the control solutions, or those receiving albumin or globulin.

No consistent microscopic changes were observed in the livers of any of the groups of protein-treated animals.

Observations on Animals Treated with Rat Serum.—Since renal enlargement and an increase in urinary protein excretion were induced by injections of heterologous proteins, it was desirable to determine whether similar effects would result from injections of homologous proteins. The experiments with

the proteins already discussed, particularly the rather preliminary studies on the specific proteins in the urine, suggested that the increased proteinuria was not dependent on the heterologous nature of the injected proteins but would be produced by homologous proteins as well. Because purified rat proteins were not available, it was decided to investigate the effects of injecting homologous serum. Some of the data from these studies have been included in the tables and charts already presented, in order to facilitate comparisons with data from other groups. However, for several reasons, separate discussion of the experiments with rat serum seemed desirable.

In the first experiment with rat serum (series 7 of Table I), the animals with the possible exception of one, appeared quite well throughout the experimental period. Some enlargement of the kidneys occurred (Chart 1), but this was of less magnitude than with the other protein solutions. At the time of autopsy, more than 12 hours after the final injection, large amounts of unabsorbed fluid were found in the peritoneal cavities of some of the animals. In experiments where solutions of gelatin, albumin, or globulin had been injected for similar periods of time, absorption had nearly always been more complete. In addition, the serum-injected rats showed evidence of peritoneal irritation with definitely thickened renal capsules, not observed in the animals receiving the 3 per cent solutions of single refined proteins. Furthermore, urine collections made on the last 2 days of the experiment, which were the only specimens collected, showed no definite increase in protein content as compared with control specimens.

It was thought that perhaps ether retained in the rat serum (the donor animals were deeply anesthetized with ether) caused progressive peritoneal irritation with a corresponding decrease in the rate of absorption of injected serum, and that this explained the fluid retention in the peritoneal cavities, the low levels of urinary protein excretion, and the absence of greater renal enlargement. A second experiment (series 8) was then performed, using rat serum which was collected from donor animals after they were rendered unconscious by anoxia in a nitrogen chamber. In this experiment, the serum-treated animals retained much of the injected serum solution in the peritoneal cavities from the beginning, excreted little urine, and became definitely ill with elevated blood urea levels. Two of the four animals died on the 4th day after becoming quite pale and cold. The other two animals were autopsied and found to have greatly enlarged kidneys. Urine collected on the 3rd and 4th days from animals of this group contained considerably increased protein levels.

Microscopic Observations on Serum-Treated Animals.—Sections from the kidneys of all but one of the animals of the first serum-treated group appeared entirely normal, with the exception perhaps that the tubular lumina contained more protein material than normal. Changes similar to those observed in the gelatin-treated animals of series 4 with the severer forms of injury, were seen in milder degree in sections of the kidneys of one rat of this first group.

When the greatly enlarged kidneys of the second serum-treated group were sectioned, a discrete band occupying the region about the corticomedullary junction, which obviously contained large amounts of calcium (Fig. 9), could be seen with the unaided eye. Microscopic examination of sections confirmed the presence of extensive tubular damage with necrosis and calcification in the zone including the deepest portion of the cortex and adjacent medullary tissue (Figs. 10 and 11). Many glomerular capsules contained protein precipitates, and the tubules in the area beneath the zone of calcification were dilated, lined by flattened epithelium, and contained large amounts of deeply staining protein material.

DISCUSSION

Only the inconclusive data of Bordley and Richards (15) and Walker *et al.* (16) are available concerning the protein content of the normal glomerular filtrate, though the constant occurrence under uniform conditions of fairly uniform levels of protein in the urine of the normal rat (17) appears to be presumptive evidence that the glomerular filtrate of that animal contains some protein. Accumulated evidence (18–21) indicates that the glomerulus is at least the chief source of the protein which appears in the urine under the various abnormal conditions which have been studied; it does not eliminate the possibility, however, that all or a part of the protein in the urine under certain circumstances may be present as a result of defective tubular reabsorption of protein normally or abnormally filtered from the glomeruli (22). It was evident from examination of the sections in the present study that the increased proteinuria which occurred with the protein injections was associated with an increased amount of protein in the glomerular filtrate. Furthermore, gelatin appeared in the urine prior to the development of the characteristic changes in the tubular cells which were presumably associated with the presence of gelatin in the cells.

Because of the chronic nature of the experiments there may have been a compensatory increase in hemoglobin levels of the protein-injected animals, and the hemoglobin levels at the end of the injection periods may not have adequately reflected the magnitude of the blood volume changes.

Proteinuria has been observed in dogs following injections of homologous plasma (23), and in human patients without renal disease following injections of homologous albumin (24). In the present experiments, an increase in urinary protein excretion was observed in some, but not in all the rats receiving homologous serum; interpretation of the results was complicated by the occurrence in some of the animals of incomplete absorption of fluid, oliguria, and renal damage.

The rather cursory study of the specific proteins in the urine of the rats seemed to indicate that increased amounts of homologous protein, as well as quantities of the heterologous protein, appeared in the urine following injec-

tions of human albumin and bovine globulin, which accumulated in the plasma, but not following gelatin which was passed into the tubular lumina to at least as great extent, but which caused less hemodilution than the other proteins. These observations might be interpreted as suggesting that the filtration of a protein present in the plasma may be increased, with no increase in the concentration of the protein, by changes in dynamics of the glomerular circulation associated with an increase in blood volume, though other explanations of the observations are conceivable.

Reversible renal enlargement, comparable in degree and rapidity of development to that observed in the present experiments following the injections of proteins, has been shown to occur in rats on diets containing high levels of various proteins, including casein (25–27). This renal hypertrophy under conditions of high protein diets has usually been considered a result of an increase in renal work associated with the metabolism and excretion of products of protein digestion. Some degree of renal enlargement due to feeding of urea itself has been observed by some investigators (28), but others have obtained essentially negative results (25). Renal injury also has occurred under certain circumstances with high protein diets (29, 30).

In the present study, renal enlargement such as occurred with the protein injections, was not observed following injections of urea or casein hydrolysate. It perhaps is possible, in spite of careful planning of the experiments, that differences in food intake, nutritional state, or hydration of tissues, might have been responsible for the difference in relative renal size which developed in the animals receiving protein hydrolysate, as contrasted with those receiving protein itself. However, there was no evidence from examination of the animals that such differences were present to an important degree.

Another explanation of the occurrence of renal enlargement with injections of proteins but not with injections of protein hydrolysate or urea, which must be considered, is that the renal enlargement might have been caused at least in part by effects on the kidney of protein molecules *per se*, perhaps more specifically by effects on the tubular cells of the increased amount of protein filtered through the glomeruli, rather than entirely by effects of products of protein digestion and metabolism reaching the kidney through the blood stream. It might be pointed out that an elevation in urinary protein excretion has been observed incidentally accompanying an increase in the dietary protein level in rats (30, 31); however, it is not intended to suggest that the increase in proteinuria was the cause of the renal hypertrophy which occurred with the high protein diets. It is difficult to understand why the protein hydrolysate injections in the present experiments did not induce some increase in proteinuria and renal enlargement through the same mechanisms as were involved with increasing the protein content of the diet.

Morphological observations by a number of European investigators, and

more recent studies by Oliver (21), Smetana and Johnson (32), Smetana (33), and Rather (34), have provided fairly conclusive evidence that at least certain proteins present in the fluid passing through the tubules may be reabsorbed by the tubular cells and accumulate to some extent within these cells. However, the results of Bott and Richards (20) indicated that not more than a small portion of the filtered protein was reabsorbed under the conditions of their experiments. The reabsorbed protein under normal conditions presumably is digested by the tubular cells (34). The observations of others on protein reabsorption, together with our own morphological observations, particularly with gelatin which was the only protein seeming to produce recognizable enlargement of the tubular cells, suggested that the renal enlargement might have been caused in some part by enlargement of individual tubular cells as a result of reabsorption and temporary accumulation within the cells of protein passing through the glomeruli, together with water associated with it. The cytoplasmic changes produced by gelatin were similar to those described by Popper (35) and Skinsnes (36) in human kidneys following gelatin administration. Furthermore, an increase in metabolic activity of the renal cells associated with an increased reabsorption and degradation of protein might have been responsible for some degree of enlargement of the kidneys.

An increase in volume of intraluminal material within the renal tubules very probably was present in a few animals in which severe renal injury occurred, and in some other gelatin-treated animals particularly during the early periods of the injections, but it appeared unlikely that changes of this nature were responsible for the renal enlargement which regularly followed the protein injections.

In the majority of animals receiving each of the 3 per cent protein solutions, there was no evidence of gross disturbances of renal function. Renal clearance studies have not been done, though such studies might yield interesting results. The changes in renal size and the alterations in the appearance of the convoluted tubules were regarded for the greatest part as reversible morphological expressions of exaggerated physiological processes, and no inflammatory, necrotic, or sclerotic changes were observed in the kidneys, either during or following the injections. Urinary protein excretion promptly returned to normal levels after injections were discontinued. It was concluded that under the conditions of the experiments, prolonged continuous proteinuria of the degree obtained with these injections in most cases did not lead to a persistent increase in glomerular permeability or to any other form of chronic or progressive renal injury.

Definite evidences of tubular injury of a severe degree were observed, however, in a few animals receiving 3 per cent gelatin, and dilatation of glomerular capsules and tubules was present in some of the animals of series 9 receiving gelatin. Whereas most of thê gelatin deposition presumably occurred in the tubular cells in the outer two-thirds of the cortex, the zone of injury was about

the corticomedullary junction. Whether this injury was a result of plugging of the tubules, or was due to some other action of gelatin, or was unrelated to any effects of gelatin on the kidney, was not determined with certainty. While the destruction of cells and proliferative changes in the dilated tubules might be considered evidence against mechanical plugging alone as the cause of the tubular lesions, at least the damaged tubules in most cases contained more protein within the lumina than usually was observed in undamaged tubules. Urine from the animals in which severe injury occurred with gelatin, unfortunately was not collected or examined. The possibility that these animals became dehydrated, due to failure to absorb part of the injected fluid, or due to failure for some reason to drink water after the injections were discontinued, in the case of the animals of series 4 which were not autopsied for several days after injection, and that dehydration contributed to the production of the injury,—as has been observed with hemoglobin (37),—must be considered. It should be noted that the concentration of protein in the urine of most of the animals in the present experiments, particularly those receiving albumin and globulin, and those of the first group receiving serum, was not as great as is often observed clinically. Furthermore, in animals with already diseased kidneys, the effects of protein injections and increased proteinuria might have been quite different from the effects observed in the present experiments employing animals with normal kidneys.

Before examining the kidneys of the second series of rats receiving homologous serum, it was considered likely that the erratic absorption of serum, and the oliguria or anuria which occurred, and perhaps also the large amounts of protein in the urine, were due to shock rather than to primary renal damage. However, the remarkable lesions which were observed were not similar to any lesions which have been attributed to disturbances of the renal circulation accompanying shock. Tubular plugging may have played a part in the production of the lesions; both the tubules and the glomerular capsules often contained large amounts of protein. The lesions were similar in certain respects to those which occurred in some of the rats receiving gelatin. Because of the great difference in the results obtained in the first and second experiments with rat serum, the profound anoxia to which the donor animals in the second experiment were subjected warrants further investigation as the possible cause of the noxious effects of the serum. It may be that the injury was due to bacterial or chemical contamination of the serum or changes in the serum subsequent to collection, and, until an attempt has been made to repeat the results, no conclusions concerning the etiology of the lesions are justified.

Finally, it might be noted that the kidneys of the protein-treated animals of the present experiments, particularly those receiving gelatin, bore certain morphological resemblances to "nephrotic" kidneys. These similarities in appearance suggested the *possibility* that, as has been concluded by a number

of investigators, certain of the changes which are seen in kidneys in diseases characterized by high levels of protein in the urine may be secondary alterations due to excessive amounts of protein passed through the glomerular membranes. Obviously no conclusions regarding the nature or the site of the fundamental disturbances in nephrosis can be drawn from these observations.

SUMMARY

Repeated intraperitoneal injections twice daily of various proteins into young rats were regularly accompanied by an increase in the protein content of the urine, significant renal enlargement, and often some degree of renal pallor. The most marked changes were induced by gelatin, followed in order by human albumin and bovine globulin. Rat serum produced similar but less conclusive changes. Similar changes were not produced by equivalent amounts of urea or casein hydrolysate.

In sections from the kidneys of animals receiving gelatin, the cells of the convoluted tubules appeared enlarged, and they contained clear "spaces" throughout the cytoplasm. The tubular cells of the animals receiving the other solutions were not obviously altered in size or shape, and the cytoplasmic changes were slight or absent. There was little evidence of increased multiplication of cells or of tubular dilatation in the kidneys of any of the groups.

Changes in concentrations of plasma proteins and hemoglobin, and the results of preliminary studies of the injected proteins in urine and renal tissue following the injections, are described and their possible significance discussed.

It appears that the renal enlargement, as well as the increase in proteinuria and the tubular alterations which followed the protein injections, might have been caused in part by effects on the kidney of protein molecules *per se*, perhaps most likely by the effects on the tubular cells of an increased amount of protein filtered through the glomerular membranes, rather than entirely by effects of products of protein digestion and metabolism reaching the kidney through the blood stream.

In the majority of animals there was no evidence from the morphological or functional studies, that the prolonged and continuous proteinuria induced by the protein injections resulted in renal damage, unless the renal enlargement, and the cytoplasmic changes which occurred regularly with gelatin, are considered evidence of damage. Renal enlargement and proteinuria promptly regressed after injections were discontinued.

Lesions characterized by severe degrees of tubular damage, possibly as a result of tubular plugging, were observed in some of the animals of one group receiving gelatin solution of the usual concentration, and dilatation of renal tubules and glomerular capsules was present in some other gelatin-treated animals autopsied after relatively brief injection periods. A description is also presented of lesions

of remarkable character which developed in the kidneys of all the animals of one small group receiving homologous serum obtained from severely anoxic donors.

The possible relationship between the renal changes in the protein-injected animals and certain alterations of the kidneys observed in diseases characterized by large amounts of protein in the urine, is considered.

BIBLIOGRAPHY

1. Baker, S. L., and Dodds, E. C., *Brit. J. Exp. Path.*, 1925, **6**, 247.
2. Forbus, W. D., Perlzweig, W. A., Parfentjev, I. A., and Burwell, J. C., Jr., *Bull. Johns Hopkins Hosp.*, 1935, **57**, 47.
3. Oliver, J., Architecture of the Kidney in Chronic Bright's Disease, New York, Paul B. Hoeber, Inc., 1939.
4. Blackman, S. S., Jr., Goodwin, W. E., and Buell, M. V., *Bull. Johns Hopkins Hosp.*, 1941, **69**, 397.
5. Ayer, G. D., and Gauld, A. G., *Arch. Path.*, 1942, **33**, 513.
6. Yuile, C. L., Gold, M. A., and Hinds, E. G., *J. Exp. Med.*, 1945, **82**, 361.
7. Lucké, B., *Mil. Surg.*, 1946, **99**, 371.
8. Baxter, J. H., *J. Nutrition*, 1947, **34**, 333.
9. Shevky, M. C., and Stafford, D. D., *Arch. Int. Med.*, 1923, **32**, 222.
10. Hiller, A., Greif, R. L., and Beckman, W. W., *J. Biol. Chem.*, 1948, **176**, 1421.
11. Hiller, A., McIntosh, J. F., and Van Slyke, D. D., *J. Clin. Inv.*, 1927, **4**, 235.
12. Phillips, R. A., Van Slyke, D. D., Dole, V. P., Emerson, K., Jr., Hamilton, P. B., and Archibald, R. M., *Bull. U. S. Army Med. Dept.*, 1943, **71**, 66.
13. Van Slyke, D. D., and Kugel, V. H., *J. Biol. Chem.*, 1933, **102**, 489.
14. Hiller, A., Plazin, J., and Van Slyke, D. D., *J. Biol. Chem.*, 1948, **176**, 1401.
15. Bordley, J., 3rd, and Richards, A. N., *J. Biol. Chem.*, 1933, **101**, 193.
16. Walker, A. M., Bott, P. A., Oliver, J., and MacDowell, M. C., *Am. J. Physiol.*, 1941, **134**, 580.
17. Addis, T., *Tr. Assn. Am. Physn.*, 1942, **57**, 106.
18. Starr, I., *J. Exp. Med.*, 1926, **43**, 31.
19. Bieter, R. N., *J. Pharmacol. and Exp. Therap.*, 1931, **43**, 407.
20. Bott, P. A., and Richards, A. N., *J. Biol. Chem.*, 1941, **141**, 291.
21. Oliver, J., *Harvey Lectures*, 1944–45, **40**, 102.
22. Dock, W., *New England J. Med.*, 1942, **227**, 633.
23. Terry, R., Hawkins, D. R., Church, E. H., and Whipple, G. H., *J. Exp. Med.*, 1948, **87**, 561.
24. Waterhouse, C., and Holler, J., *J. Clin. Inv.*, 1948, **27**, 560.
25. Osborne, T. B., Mendel, L. B., Park, E. A., and Winternitz, M. C., *J. Biol. Chem.*, 1927, **71**, 317.
26. MacKay, E. M., MacKay, L. L., and Addis, T., *Am. J. Physiol.*, 1928, **86**, 459.
27. Addis, T., Glomerular Nephritis, Diagnosis and Treatment, New York, The Macmillan Co., 1948.
28. MacKay, L. L., MacKay, E. M., and Addis, T., *J. Nutrition*, 1931, **4**, 379.
29. Newburg, L. H., *Arch. Int. Med.*, 1919, **24**, 359.
30. Moise, T. S., and Smith, A. H., *Arch. Path.*, 1927, **4**, 530.

31. Addis, T., MacKay, E. M., and MacKay, L. L., *J. Biol. Chem.*, 1926, **71,** 157.
32. Smetana, H., and Johnson, F. R., *Am. J. Path.*, 1942, **18,** 1029.
33. Smetana, H., *Am. J. Path.*, 1947, **23,** 255.
34. Rather, L. J., *J. Exp. Med.*, 1948, **87,** 163.
35. Popper, H., *Arch. Surg.*, 1945, **50,** 34.
36. Skinsnes, O. K., *Surg., Gynec. and Obst.*, 1947, **85,** 563.
37. Lalich, J. J., *J. Exp. Med.*, 1948, **87,** 157.

EXPLANATION OF PLATES

PLATE 32

The photographs were made by Mr. R. F. Carter.

FIG. 1. Comparing left kidney from gelatin-treated rat (above) with homolateral kidney from comparable saline-injected control animal (below). The organs had not been fixed. The animals were from series 1 and received injections for 6 weeks. Note enlargement, particularly in the cortical portion, and paleness of the kidney from the gelatin-injected animal. Weights are as follows:—

Control rat: weight of kidneys, 1.85 gm.; weight of rat, 146 gm.; $\frac{\text{Weight of kidneys}}{\text{Weight of rat}} \times 100 = 1.27$

Gelatin rat: weight of kidneys, 2.50 gm.; weight of rat, 140 gm.; $\frac{\text{Weight of kidneys}}{\text{Weight of rat}} \times 100 = 1.79$

Magnification $\times$ 2.

FIG. 2. Comparing left kidney from albumin-treated rat (above) with control kidney (below). The animals were from series 2, and received injections for 5 weeks. Note enlargement and cortical thickening in kidney of albumin-treated animal. Weights are as follows:—

Control rat: weight of kidneys, 1.54 gm.; weight of rat, 126 gm.; $\frac{\text{Weight of kidneys}}{\text{Weight of rat}} \times 100 = 1.22$

Albumin rat: weight of kidneys, 2.10 gm.; weight of rat, 126 gm.; $\frac{\text{Weight of kidneys}}{\text{Weight of rat}} \times 100 = 1.67$

Magnification $\times$ 1.5.

FIG. 3. Comparing right kidneys from comparable rats of series 6 receiving various solutions. Animals received injections for 9 days. Top row, left to right:

Gelatin: weight of kidneys, 1.10 gm.; weight of rat, 64 gm.; $\frac{\text{Weight of kidneys}}{\text{Weight of rat}} \times 100 = 1.72$

Albumin: weight of kidneys, 1.03 gm.; weight of rat, 66 gm.; $\frac{\text{Weight of kidneys}}{\text{Weight of rat}} \times 100 = 1.56$

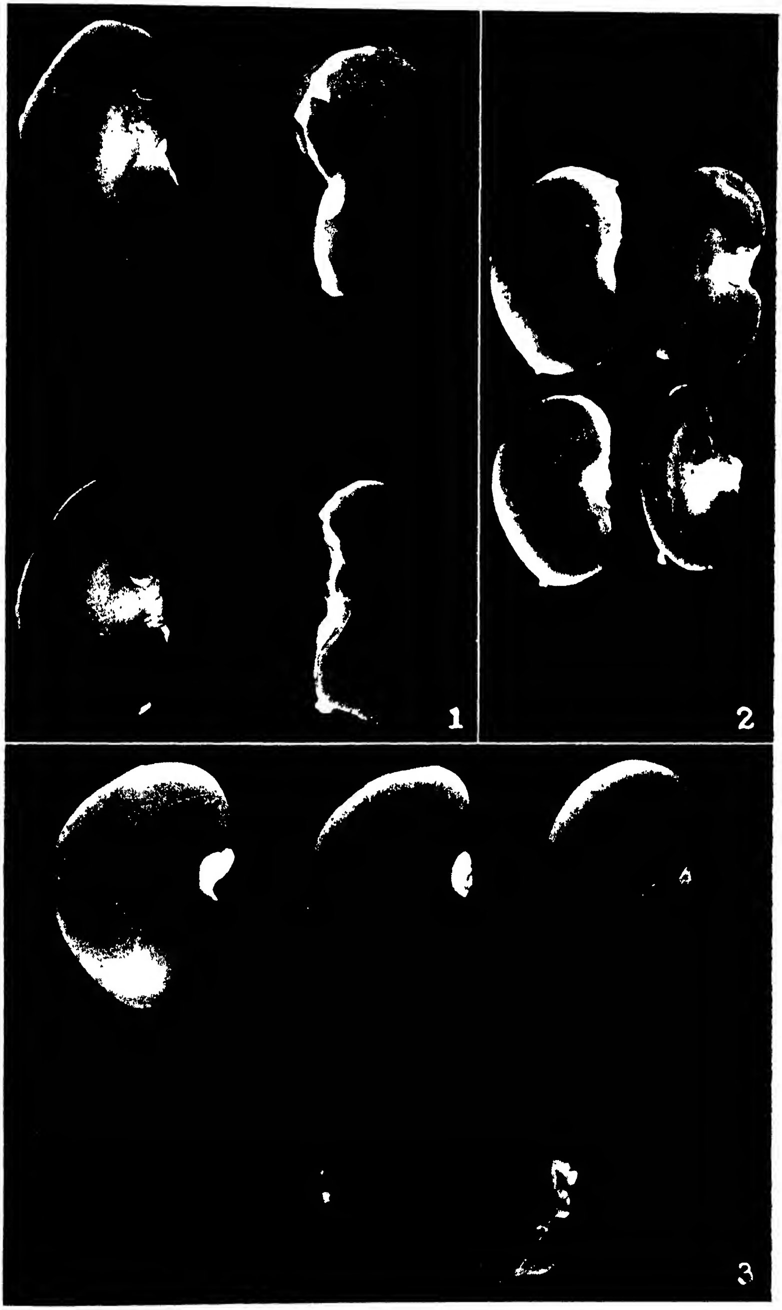
Globulin: weight of kidneys, 1.03 gm.; weight of rat, 66 gm.; $\frac{\text{Weight of kidneys}}{\text{Weight of rat}} \times 100 = 1.56$

Bottom row, left to right:

Casein hydrolysate: weight of kidneys, 0.78 gm.; weight of rat, 64 gm.; $\frac{\text{Weight of kidneys}}{\text{Weight of rat}} \times 100 = 1.22$

Saline: weight of kidneys, 0.78 gm.; weight of rat, 64 gm.; $\frac{\text{Weight of kidneys}}{\text{Weight of rat}} \times 100 = 1.22$

Magnification $\times$ 4.



(Baxter and Cotzias: Parenteral protein and the kidney)

PLATE 33

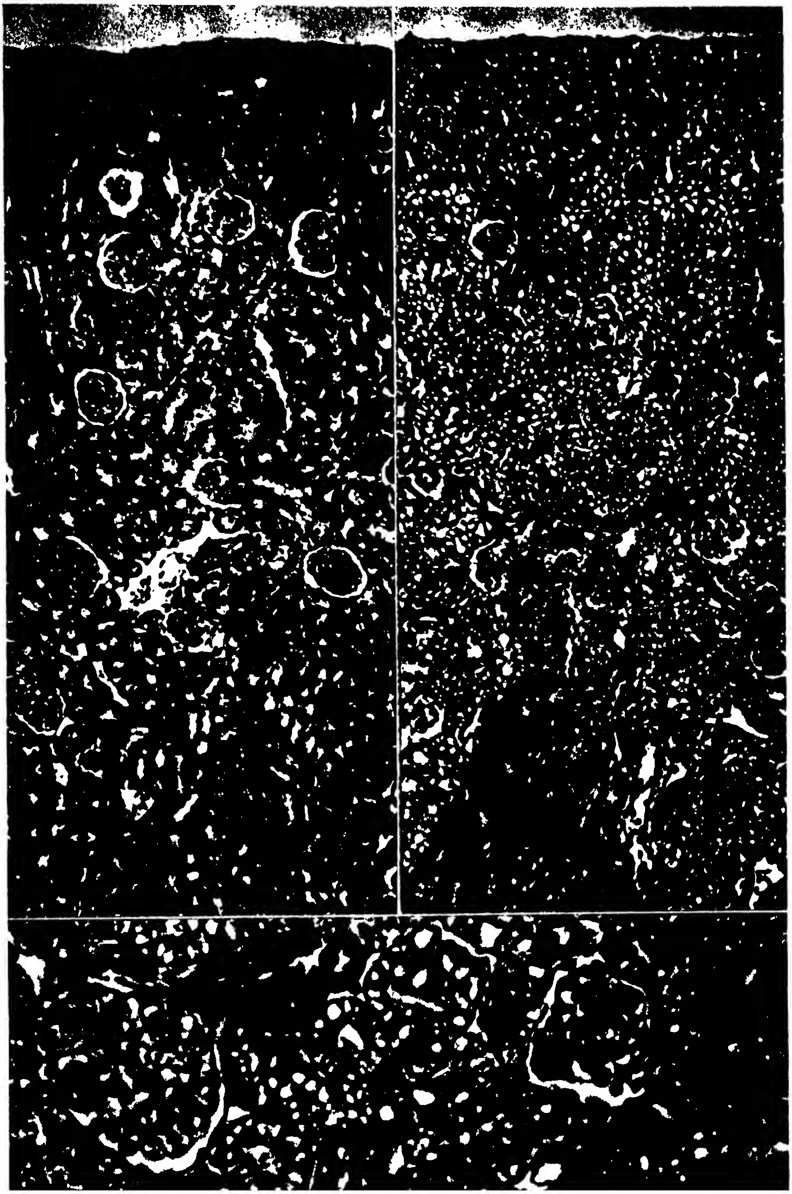
All the sections were stained with hematoxylin and eosin.

The photographs were made by Mr. J. A. Carlile.

FIG. 4. Sagittal section through a control kidney of same group as one shown in Fig. 1. × 120.

FIG. 5. Similar section through kidney of comparable gelatin-treated animal of series 1. Note enlargement of cells of convoluted tub les, and what appear to be clear spaces within the cytoplasm of the cells. × 120.

FIG. 6. Higher magnification of section shown in Fig. 5, showing cytoplasmic alterations in greater detail. × 350.



(Baxter and Cotzias: Parenteral protein and the kidney)

PLATE 34

All the sections were stained with hematoxylin and eosin.

The photographs were made by Mr. C. F. Reather, Johns Hopkins School of Medicine.

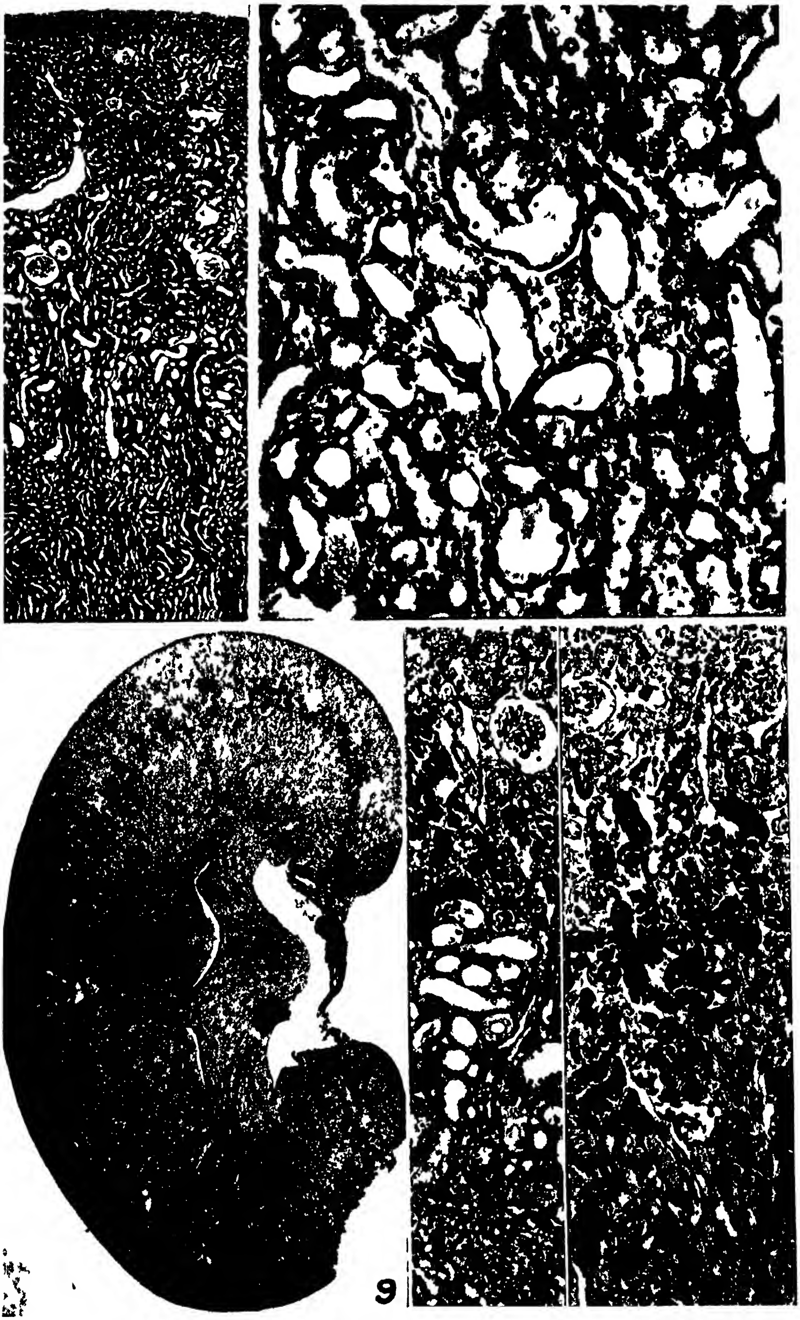
FIG. 7. Sagittal section through kidney of gelatin-treated animal of series 4. This animal received only three injections of gelatin at 12 hour intervals, and was autopsied 54 hours after the last injection. At a higher magnification clear spaces could still be seen in the cells of the convoluted tubules in the outer two-thirds of the cortex. Deeper in the cortex and in the outer portion of the medulla, many glomerular capsules and tubules are dilated. This section shows the most extensive damage observed in any of the kidneys of the animals receiving the 3 per cent solution of gelatin. × 40.

FIG. 8. Higher magnification of the section shown in Fig. 7, showing details of the tubular alterations in the zone of the most extensive injury. Many of the tubules are dilated, lined by flattened and markedly basophilic epithelium, and contain pink-staining protein material Mitotic figures and other nuclear changes indicative of active cellular proliferation were also present. × 200.

FIG. 9. Sagittal section from kidney of a serum-treated animal of series 8, which became obviously ill and was autopsied on the 5th day. This animal was anuric through a considerable part of the injection period but excreted a moderate amount of urine containing large amounts of protein during the 24 hours before autopsy. The blood urea level was approximately 100 mg. per cent at the time of autopsy. The zone of tubular necrosis and calcification, and the zone of tubular dilatation, are shown well. × 8.

FIG. 10. Higher magnification of section shown in Fig. 9. The zone of calcification can be seen about the region of the corticomedullary junction. Adjacent to and toward the pelvis from this calcified zone, there is the zone of dilated tubules, many of which contain protein. Many tubules far down toward pelvis, which were not dilated, also were full of protein material. × 100.

FIG. 11. A similar section from kidney of the other serum-treated animal of series 8 which was autopsied on the 5th day. In this kidney most of the glomerular capsules contained protein precipitates. × 100.



(Baxter and Cotzias: Parenteral protein and the kidney)

[Reprinted from THE JOURNAL OF PARASITOLOGY, August, 1949,
Vol. 35, No. 3, pp. 411–416]

THE COURSE OF INFECTION OF PLASMODIUM LOPHURAE IN CHICK EMBRYOS

BY R. BARCLAY McGHEE

(*From the Department of Animal and Plant Pathology of the The Rockefeller Institute for Medical Research, Princeton, New Jersey*)

(Received for publication, January 27, 1949)

INTRODUCTION

It is known that the age of the host exerts a decided influence on infections with *Plasmodium lophurae* in chickens (Coggeshall, 1938; W. H. and L. G. Taliaferro, 1940; Terzian, 1941), but no studies have been made of this parasite in chick embryos. Although infections have been established in duck embryos (Wolfson, 1940; Stauber and Van Dyke, 1945), no attempt has been made previously to follow the course of infection in individual embryos. In order to further determine the effect of age upon susceptibility and to make a detailed study of the course of infection, chick embryos were inoculated with erythrocytic stages of *P. lophurae* and daily studies made on infected individuals of various age groups.

Materials and Methods

The strain of *P. lophurae* used in this study was derived from strain 12A carried in this laboratory through Rhode Island Red chicks. Both White Leghorn and Rhode Island Red chick embryos served as experimental hosts. Inoculations in embryos were made following the method of Eichorn (1940). Blood for serial passages was withdrawn from one of the large allantoic vessels (Beveridge and Burnet, 1946). As much as 0.4 cc of blood has been obtained in this fashion without resulting in the death of the embryo. The greatest amount of blood obtained from a single embryo was 0.75 cc. Inocula consisted of 3×10^7 parasites per embryo unless otherwise indicated.

Daily blood smears were made in the following manner: A large allantoic vein was located and the overlying shell marked with a series of short lines at right angles to the course of the vein. Succeeding lines were drilled daily. The drill was allowed to just penetrate the inner shell membrane, thus abrading the vein to the extent that sufficient blood for a smear could be drawn into a capillary pipette. If this procedure was carefully followed no hemorrhage resulted. The exposed area was sealed with celloidon and the egg was returned to the incubator.

Blood smears were stained with Giemsa after fixation with methyl alcohol. Semilogarithmic scales were used in graphs to accentuate the preliminary rise of infection and to indicate any minor relapses. Parasite counts during the

course of infection were stated at the number per 10,000 red blood cells, with a 10 per cent probable error or less for values over 150 parasites per 10,000 red cells, of 15 per cent or less for those between 50 and 150, and of 20 per cent or less for those below 50 (Gingrich, 1932).

EXPERIMENTAL RESULTS

Three hundred twenty chick embryos 7 through 15 days of age, representing 15 passages, have been intravenously inoculated with erythrocytic stages of *P. lophurae*. Mortality rates resulting from trauma differed according to the age of the embryo at inoculation. Although only 5 seven-day embryos were injected none died from parasite introduction. The mortality rate for 107 ten day embryos was 35 per cent, but in 100 embryos inoculated at the close of the work, the rate dropped to 13 per cent. Two per cent of 108 embryos inoculated at 14 days died within 2 days. All embryos surviving beyond this time became infected.

The average time to death due to malaria varied in relation to the number of parasites introduced. Embryos died from infection as early as 4 days or survived as long as 10 days. Only two embryos, both of which had been inoculated at 15 days, survived hatching. Both died within one hour following emergence. In the initial and first passages, parasite numbers dropped in certain embryos on the first or second day. In embryo 12, for example, the parasitemia dropped from 400 on the first day to 20 on the second, followed by a quick recovery and rapid rate of reproduction (Fig. 1). In succeeding passages, parasite numbers did not decline but increased at a rapid rate until the death of the embryo, whether inoculated at 10 or 15 days incubation. Parasitemias reaching 22,500 parasites per 10,000 red blood cells were recorded in embryos inoculated at 15 days incubation and 11,200 parasites per 10,000 red blood cells in embryos inoculated at 10 days. No crisis appeared. Infections were similar to *P. gallinaceum* infections in chick and duck embryos (McGhee, 1949) and progressed until the death of the host.

The rate of parasite reproduction was slightly greater in older embryos (Fig. 2A). Within age groups, however, parasite increases were uniform, the peak of parasitemia being dependent on the inoculum. For example, in group of 15 embryos each given 3×10^7 parasites the maximum and minimum per day throughout the life of the embryo differed but slightly from the mean parasite count (Fig. 3).

Although there was no great difference in the rate of parasite reproduction in embryos inoculated at various ages, the blood picture of the host was observed to vary with this factor. The blood of normal chick embryos of 10 days incubation has 38 per cent immature cells composed chiefly of polychromatophil erythroblasts, a few basophil erythroblasts and large lymphocytes (the lymphoid haemocytoblasts of Danchakoff, 1916). About 9 per cent of the blood cells were primitive erythroblasts. In the ensuing days the number of immature cells

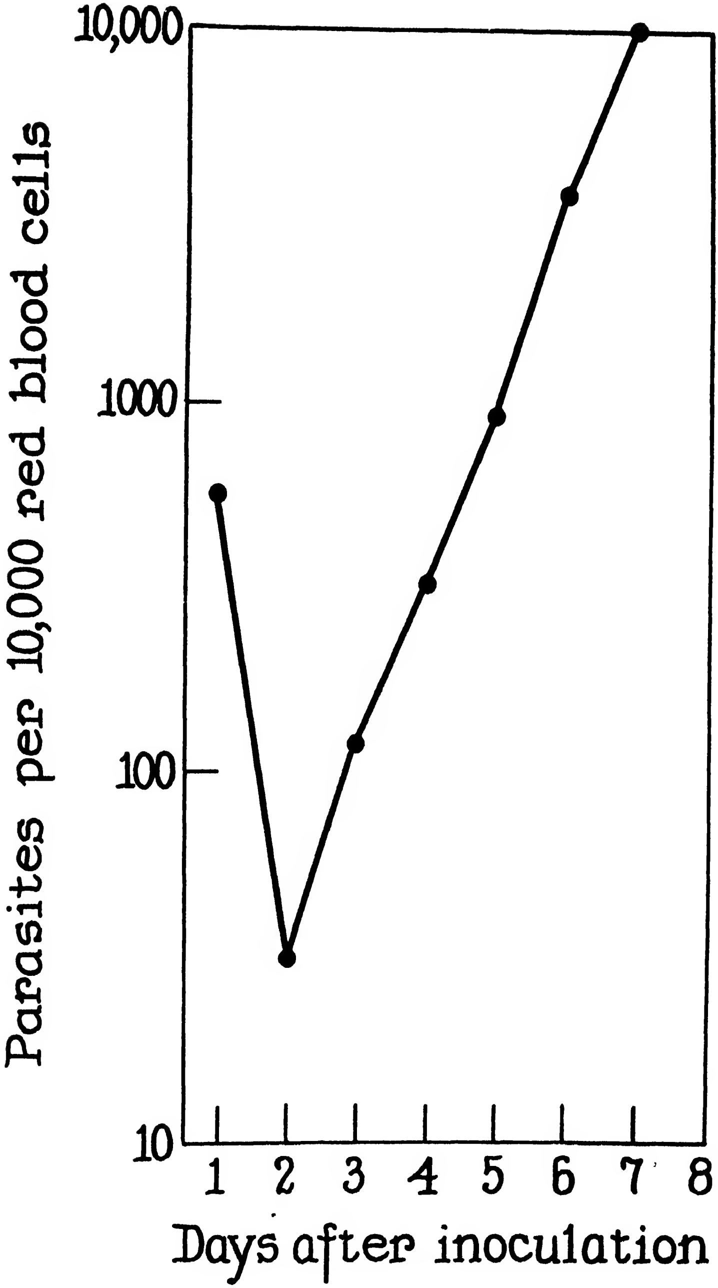


FIG. 1. Parasite counts of embryo 12 (1st passage) to show the preliminary drop in parasite numbers.

diminished until, at the 20th day, the picture was essentially that of a hatched chick (Fig. 2B).

In embryos infected at 10 days the young cell count remained high, increasing slightly just prior to death (Fig. 2B). Throughout the period of infection some

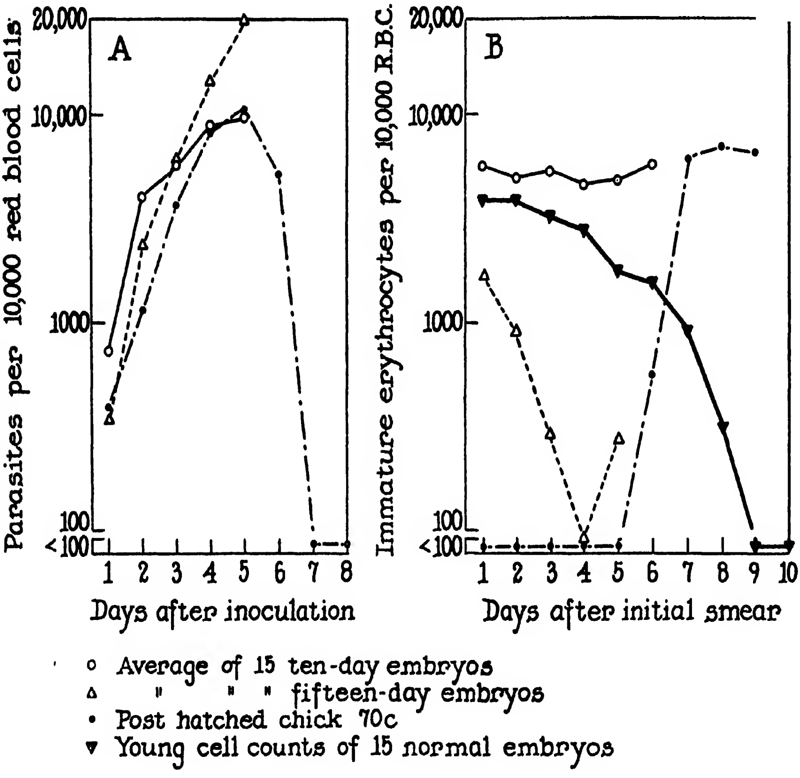


FIG. 2. A, The course of the parasitemia in a chick inoculated when 14 days old and in embryos inoculated at 10 and at 15 days of incubation with parasites from the 5th embryo passage; B, The changes in the proportions of immature erythrocytes in the same animals during the course of their infections and, for comparison, a curve showing the normal downward trend of the number of young red cells in uninfected embryos during the 11th to 21st days of incubation (obtained from normal embryos some of which were bled daily while others were sacrificed at various days).

immature definitive erythrocytes were infected, the number rising in one embryo to 56 per cent. Ninety-two per cent of the primitive erythroblasts were infected in certain embryos at the height of infection. Although as high as six parasites have been found in a single primitive erythroblast, only one parasite has been found segmenting. The parasites stained more deeply, appeared more

dense, and often had large vacuoles in the cytoplasm. The high young cell count was not due to daily bleeding since in uninfected embryos from which

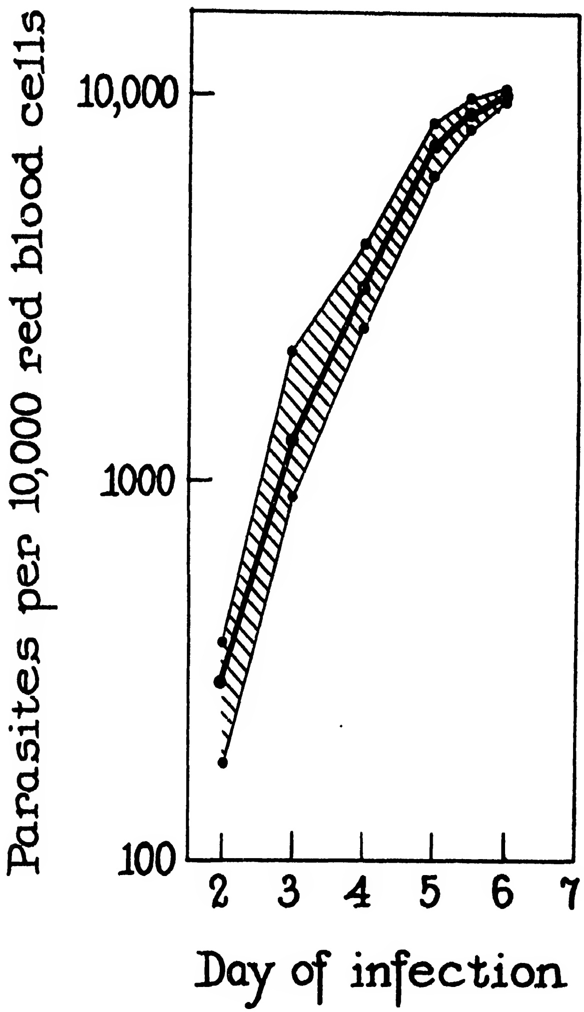


FIG. 3. The mean (heavy line) and range (shaded area) of 10 embyros inoculated at 10 days with 3×10^7 parasites.

smears were made daily the blood picture was the same as that recorded for individuals bled on only one day. Young cell counts in embryos inoculated at 15 days were low initially and continued to drop in numbers until just prior to death (Fig. 2B). The number of primitive erythroblasts was too low for accurate counts.

DISCUSSION

P. lophurae infections in chick embryos were distinguished by a rapidly increasing parasitemia and the absence of any crisis. The greater susceptibility to infection further extends the findings of Coggeshall (1938), W. H. and L. G. Taliaferro (1940), and Terzian (1941) on the influence of host age upon susceptibility. It is of interest to note that the smallest inoculum produced a high, albeit a prolonged, infection invariably resulting in the death of the embryo. These results emphasize the lack of resistance in embryos and concur with the findings of Murphy (1914) and Grasset (1929).

P. lophurae has been considered a parasite which, to a great extent, invades only mature erythrocytes (Terzian, 1941). Infections in younger embryos indicated that cell age is an inefficient barrier to invasion, except possibly on the initial passage, and confirmed the observations of Stauber and Van Dyke (1945) who reported 76 per cent of the primitive erythroblasts in duck embryos infected with *P. lophurae*. The presence of parasites in these cells would seem to indicate an adaptation, but if an adaptation was present it was not in the nature of *Dauermodifikationen* since subinoculation into hatched chicks produced an infection typical for the parasite (Fig. 2A). It is more reasonable to assume that the primitive erythroblasts more nearly resembled the mature erythrocyte, and were utilized by the parasite in preference to the cells of the lower definitive erythrocytic series.

The difference in the per cent of young cells in embryos inoculated at various ages might account for the difference in reproduction rates. As seen in figure 2B the stimulus applied by infection caused the young cell count to remain high in 10 day embryos. In older embryos, there were sufficient mature erythrocytes to afford a greater selection of host cells without causing destruction to the extent of interposing a burden on the embryo blood system until just prior to death. It seems logical to assume, therefore, that although parasites are capable of infecting young cells, the chances of survival of merozoites in a medium composed largely of such young cells would be diminished.

The uniformity between infections in different individuals was quite different from that seen in post-hatched chicks, in which fluctuations in parasite curves are quite common. The uniformity in embryos perhaps reflected the more controlled conditions of movement, darkness and nutrition.

SUMMARY

P. lophurae infections in chick embryos were characterized by a rapid increase in parasite numbers, the absence of a crisis, and the death of the host. Infections increased more rapidly in older than in younger embryos, although the infections in any given age group were distinctly uniform. Following introduction in 10 day embryos the young red cell count remained high, in 15 day embryos it dropped until just before death. A high percentage of primitive erythroblasts and a certain proportion of immature definitive erythrocytes were parasitized.

REFERENCES

BEVERIDGE, W. I. B. AND BURNET, F. M. 1946 The cultivation of viruses and rickettsiae in the chick embryo. Medical Research Council. Special Report Series No. 256.

COGGESHALL, L. T. 1938 *Plasmodium lophurae* a new species of malaria parasite pathogenic for the domestic fowl. Am. J. Hyg. **27**: 615–618.

DANCHAKOFF, V. 1916 Equivalence of different haemopoietic anlages I. Am. J. Anat. **20**: 255–328.

EICHORN, E. A. 1940 A technique for the intravenous inoculation of chick embryos. Science **29**: 245–246.

GINGRICH, W. 1932 Immunity to superinfection and cross immunity in malarial infections in birds. J. Prev. Med. **6**: 197–246.

GRASSET, E. G. 1929 A comparative study of the aptitude of the higher animal organism to acquire immunity throughout the vital cycle and the relation of this aptitude to heredity transmission. Sth. Afr. Inst Med. Res. Publ. No. 24.

McGHEE, R. B. 1949 The course of infection of *Plasmodium gallinaceum* in duck embryos. J. Infect. Dis. **84**: 98–104.

MURPHY, J. B. 1914 Studies in tissue specificity II and III. J. Exp. Med. **17**: 482–485.

STAUBER, L. A. AND VAN DYKE, A. B. 1945 Malarial infections in the duck embryo. Proc. Soc. Exp. Biol. and Med **58**: 125.

TALIAFERRO, W. H. AND TALLIAFERRO, L. G. 1940 Active and passive immunity in chickens against *Plasmodium lophurae*. J. Infect. Dis. **66**: 153–165.

TERZIAN, L. A. 1941 Studies on *Plasmodium lophurae*. A malarial parasite in the fowl. II. Pathology and the effect of experimental conditions. Am. J. Hyg. **33**: 33–53.

WOLFSON, F. 1940 Successful cultivation of avian *Plasmodia* in duck embryos. Am. J. Hyg. **32**: 60–61.

INDEX TO AUTHORS

INDEX TO SUBJECTS